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*SUGAR NUCLEOTIDES IN THE INTERCONVERSION OF CARBOHYDRATES IN HIGHER PLANTS**

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Read before the Academy, April 27, 1959

Sugar nucleotides are compounds containing sugars or sugar derivatives esterified through the glycosidic hydroxyl to the second phosphate of a nucleoside 5′-pyrophosphate. They are widely distributed in nature, and in the last decade have been shown to occupy a central position in carbohydrate metabolism.[1,2] A number of sugar interconversions occur only when the sugars are linked to a nucleoside diphosphate.[3–9] The sugar nucleotides also function as glycosyl donors in many transglycosylation reactions, giving rise to a variety of oligosaccharides,[10–13] glycosides,[14–16] and polysaccharides.[17–23] The complex saccharides that have been synthesized by the transglycosylation process involving nucleotides include sucrose and callose (β-1,3-glucan) in higher plants,[23] glycogen in animal tissues,[20–22] and chitin in *Neurospora crassa*.[18]

In 1951 Leloir *et al.*[24,25] were the first to isolate a sugar nucleotide, uridine diphosphate (UDP) D-glucose (Fig. 1) from yeast in connection with their study of

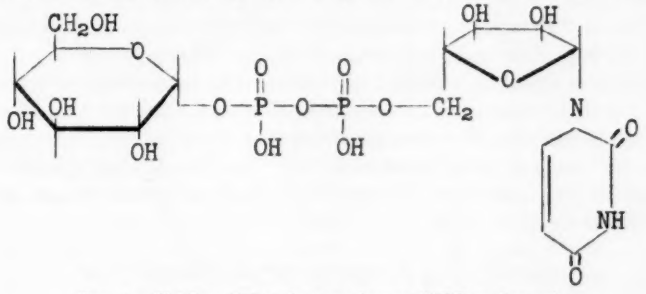


FIG. 1.—Uridine diphosphate D-glucose (UDP-D-glucose).

D-galactose utilization. They demonstrated that a cofactor was required for the transformation of α-D-glucose 1-phosphate to α-D-galactose 1-phosphate, and this cofactor was identified as UDP-D-glucose. Since that time steadily increasing numbers of sugar nucleotides of the UDP-D-glucose type have been detected or isolated from animals, plants, and bacteria.

The sugar nucleotides, containing aldoses, amino sugars, and uronic acids as the sugar moiety, have a similar chemical structure. Most of the sugar nucleotides

contain uridine as a moiety of the nucleotide molecule. However, two sugar nucleotides in which guanosine is the nucleoside component (guanosine diphosphate D-mannose[26] and guanosine diphosphate L-fucose[27, 28]), and two sugar alcohol nucleotides in which cytidine is the nucleoside component (cytidine diphosphate glycerol and cytidine diphosphate ribitol) are known.[29, 30]

Several UDP-sugars in which the sugar moiety is linked to an additional non-carbohydrate residue have been described. UDP-N-acetyl-D-glucosamine phosphate and UDP-N-acetyl-D-galactosamine sulfate have been isolated from hen's oviduct by Strominger,[31] and three UDP-N-acetylmuramic acid compounds, two of which contained amino acids, were detected by Park in cells of *Staphylococcus aureus* exposed to penicillin.[32, 33] Recently evidence has been presented for the presence of a UDP-linked neuraminic acid peptide in culture filtrates of a strain of *E. coli*.[34]

We have been studying the reactions involving the formation and interconversion of sugar nucleotides in higher plants, particularly the pathways that lead to the formation of complex saccharides.

Isolation of Sugar Nucleotides.—Working with mung bean (*Phaseolus aureus*) seedlings Ginsburg *et al.*[35] isolated and identified a mixture of UDP-D-glucose, UDP-D-galactose, UDP-D-xylose, and UDP-L-arabinose by procedures based on those of Cabib *et al.*[36] (ion exchange chromatography followed by paper electrophoresis and paper chromatography). Later Solms and Hassid[37] isolated UDP-N-acetyl-D-glucosamine and UDP-D-glucuronic acid from the same plants. The two pentose nucleotides, UDP-D-xylose and UDP-L-arabinose, were found for the first time in nature in mung beans.

Synthesis of Sugar Nucleotides by Pyrophosphorylases.—Trucco[38] showed that UDP-D-glucose was formed when a mixture of adenosine triphosphate (ATP) and UDP was incubated in the presence of α-D-glucose 1-phosphate and a preparation from yeast. He postulated that the UDP was first phosphorylated to give uridine triphosphate (UTP), which subsequently reacted with the α-D-glucose 1-phosphate, to give UDP-D-glucose and pyrophosphate (PP). Munch-Petersen *et al.*[39] showed that UDP-D-glucose could be formed by yeast extracts from α-D-glucose 1-phosphate and UTP, with the concomitant liberation of pyrophosphate. Using isotopically labeled pyrophosphate, they demonstrated that the pyrophosphate group arises from the two terminal phosphates of UTP. The reaction can therefore be considered to involve a transfer of a uridyl group from pyrophosphate to a sugar 1-phosphate molecule:[40]

$$\text{Uridine-P}\overset{*}{\text{P}}\overset{*}{\text{P}} + \text{P-sugar} \rightleftharpoons \text{uridine-PP-sugar} + \overset{*}{\text{P}}\overset{*}{\text{P}}$$

Using a number of sugar 1-phosphates and UTP labeled with P^{32} in the two terminal phosphates, Neufeld *et al.*[4] found that partially purified mung bean pyrophosphorylases did not catalyze an exchange of P^{32} between this nucleotide and pyrophosphate unless sugar 1-phosphate was present. This indicates that no free uridyl-enzyme complex is formed and that the pyrophosphorolysis reaction occurs by a mechanism involving a simultaneous attachment of both uridyl donor and acceptor to the enzyme surface. The mechanism of the reaction can be visualized as follows:

$$\text{Uridine-P}\overset{**}{\text{P}}\overset{**}{\text{P}} + \text{P-sugar} + \text{Enzyme} \rightleftharpoons \text{Enzyme-}\left[\text{Uridine-P}\begin{matrix}\overset{**}{\text{P}} \\ \text{P-sugar}\end{matrix}\right]$$
$$\rightleftharpoons \text{Enzyme} + \text{Uridine-PP-sugar} + \overset{**}{\text{P}}\overset{**}{\text{P}}$$

Munch-Petersen[41] has shown that yeast UDP-D-glucose pyrophosphorylase likewise does not catalyze any exchange between UTP and pyrophosphate in the absence of α-D-glucose 1-phosphate.

It has been shown in our laboratory that extracts from higher plants contain pyrophosphorylases capable of catalyzing the reversible formation of all the sugar nucleotides that have been isolated from mung bean seedlings. Thus in the presence of UTP and extracts from a number of plants, UDP derivatives of D-glucose, D-galactose, D-xylose, and L-arabinose were formed by this reaction from α-D-glucose 1-phosphate, α-D-galactose 1-phosphate, α-D-xylose 1-phosphate and β-L-arabinose 1-phosphate, respectively:

$$\text{UTP} + \alpha\text{-D-glucose 1-P} \rightleftharpoons \text{UDP-D-glucose} + \text{PP}$$

$$\text{UTP} + \alpha\text{-D-galactose 1-P} \rightleftharpoons \text{UDP-D-galactose} + \text{PP}$$

$$\text{UTP} + \alpha\text{-D-xylose 1-P} \rightleftharpoons \text{UDP-D-xylose} + \text{PP}$$

$$\text{UTP} + \beta\text{-L-arabinose 1-P} \rightleftharpoons \text{UDP-L-arabinose} + \text{PP}$$

It was reported in the original publication[4] that α-L-arabinose 1-phosphate could also serve as substrate for the pyrophosphorylase reaction. We have since been unable to repeat this result and believe that the sample of α-L-arabinose 1-phosphate used was impure, being contaminated with the β-anomer that is reactive in this system.

Only the α-D-anomers of the hexose 1-phosphates undergo the pyrophosphorylase reaction. But since the configuration of the β-L-arabinose 1-phosphate around its C-1 atom is the same as that of the α-anomer of the D-series, it is the β-rather than the α-anomer of L-arabinose 1-phosphate that reacts in this system.

Ginsburg[42] succeeded in purifying UDP-D-glucose pyrophosphorylase from mung bean seedlings approximately 800-fold. The purified preparation appeared to be specific for UDP-D-glucose. The pyrophosphorylase activities toward the other sugar nucleotides have thus far not been separated from each other.

Evidence was later presented by Feingold *et al.*[43] that pyrophosphorylase(s) are present in mung bean extracts capable of catalyzing the reversible formation of the two uronic acid nucleotides, one from UTP and α-D-glucuronic acid 1-phosphate, and another from UTP and α-D-galacturonic acid 1-phosphate according to the following reactions:

$$\text{UTP} + \alpha\text{-D-glucuronic acid 1-P} \rightleftharpoons \text{UDP-D-glucuronic acid} + \text{PP}$$

$$\text{UTP} + \alpha\text{-D-galacturonic acid 1-P} \rightleftharpoons \text{UDP-D-galacturonic acid} + \text{PP}$$

There is no information at present as to whether the pyrophosphorylase acting in the above reactions is the same enzyme or whether a different enzyme is involved in each case. However, it is known that the UDP-uronic acid pyrophosphorylase(s) are distinct from other UDP-sugar pyrophosphorylases present in plants.[42]

Smith and Mills,[44] investigating the action of pyrophosphorylases on UDP-D-glucuronic acid, found that neither the yeast enzyme nor that from liver nuclei had any action. The results were negative with UTP and α- or β-D-glucuronic acid 1-phosphate. In animal cells, UDP-D-glucuronic acid is formed by oxidation of UDP-D-glucose to UDP-D-glucuronic acid, as shown by Strominger *et al.*:[45, 46]

$$\text{UDP-D-glucose} + 2\text{DPN}^+ \rightarrow \text{UDP-D-glucuronic acid} + 2\text{DPNH} + 2\text{H}^+$$

The same reaction was shown to occur in bacteria,[47] in pea seedlings,[48] and in mung beans.[49] Thus, plants possess two mechanisms by which UDP-D-glucuronic acid may be formed: one by oxidation of UDP-D-glucose, and another by transfer of the uridyl group from UTP to α-D-glucuronic acid 1-phosphate.

A pyrophosphorylase acting on UDP-N-acetyl-D-glucosamine has been found in liver nuclei[44] and in yeast.[17, 50] Feingold *et al.*[51] demonstrated that such an enzyme is also present in plants. The following reversible reaction is catalyzed by a preparation from mung beans:

$$\text{UTP} + \text{N-acetyl-}\alpha\text{-D-glucosamine 1-P} \rightleftharpoons \text{UDP-N-acetyl-D-glucosamine} + \text{PP}$$

The presence of UDP-N-acetyl-D-glucosamine and of the enzyme that catalyzes its formation in plants was unexpected. N-acetyl-α-D-glucosamine is known to exist in polymeric form in chitin, a compound that constitutes the exoskeletons of crustaceans and the cell walls of fungi, and in heteropolysaccharides such as hyaluronic acid or mucopolysaccharides.[52] Until recently higher plants were considered to be devoid of N-acetyl-α-D-glucosamine either in free or polymeric form. However, it has been lately reported that certain plants and especially pollen grains contain a substance which on hydrolysis produces D-glucosamine.[53, 54] Free amino sugars have been found in pineapple plants.[55] Carter *et al.*[55a] have recently furnished definite evidence for the presence of D-glucosamine as a component of glycolipides of higher plants, by isolating the aminosugar from the hydrolysis products of the glycolipides as the crystalline D-glusosamine hydrochloride.

C-4 Epimerases.—A UDP-D-galactose 4-epimerase (galactowaldenase) that catalyzes the interconversion of UDP-D-glucose and UDP-D-galactose is known to exist in yeast, bacteria, and animals.[3] Recently a similar UDP-D-galactose 4-epimerase and two other C-4 epimerases, i.e., UDP-L-arabinose 4-epimerase and UDP-D-galacturonic acid 4-epimerase, were found in mung beans.[4, 5] An epimerase which catalyzes the conversion of UDP-D-glucuronic acid to UDP-D-galacturonic acid was also demonstrated by Smith *et al.*[55b] to be present in a strain of *Pneumococcus*. All these enzymes cause epimerization of the hydroxyl group on the 4th carbon atoms of the respective sugar molecules.

Data obtained from experiments with wheat seedlings into which D-galactose 1-C^{14} was introduced and allowed to metabolize, indicated that conversion of D-galactose to D-glucose occurred without degradation of the hexose chain.[56] These data, together with the identification of D-galactose 1-phosphate in Canna leaves, suggested that D-galactose was converted to D-glucose in higher plants through a mechanism involving UDP-D-galactose 4-epimerase.

Working with mung bean seedling extracts fractionated with ammonium sulfate (50 to 70 per cent), Neufeld *et al.*[4] showed that a mixture of UDP-D-glucose and UDP-D-galactose was obtained when UDP-D-glucose was incubated with this

enzyme fraction, demonstrating the presence of a UDP-D-galactose 4-epimerase. This fraction also contained UDP-L-arabinose 4-epimerase activity. The UDP-D-galactose 4-epimerase activity has not been separated from the UDP-L-arabinose 4-epimerase activity. Yeast UDP-D-galactose 4-epimerase does not catalyze the formation of UDP-D-xylose from UDP-L-arabinose.

Neufeld *et al.*[5] showed that particulate preparations from these plants contain a UDP-D-galacturonic acid 4-epimerase capable of catalyzing the interconversion of UDP-D-glucuronic acid and UDP-D-galacturonic acid:

$$\text{UDP-D-glucuronic acid} \rightleftharpoons \text{UDP-D-galacturonic acid}$$

The particulate preparation contained UDP-L-arabinose 4-epimerase as well as UDP-D-galacturonic acid 4-epimerase activity. When this preparation was extracted with digitonin, the UDP-D-galacturonic acid 4-epimerase activity was lost, while the UDP-L-arabinose 4-epimerase activity was retained, indicating that the two activities are associated with different enzymes.[57]

D-Glucuronic, D-Galactose and L-Arabinose Kinases.—Most kinases catalyze the phosphorylation of the primary hydroxyl group of sugars. However, a kinase that phosphorylates D-galactose on the glycosidic hydroxyl is known to exist in yeast, animals, and bacteria.[3] We have now found that enzyme preparations from mung bean seedlings, in the presence of ATP and magnesium chloride, are capable of phosphorylating D-glucuronic acid, L-arabinose, and D-galactose, causing the formation of α-D-glucuronic acid 1-phosphate, β-L-arabinose 1-phosphate, and α-D-galactose 1-phosphate, respectively, and presumably ADP.[58, 59]

$$\text{D-Glucuronic acid} + \text{ATP} \rightarrow \alpha\text{-D-glucuronic acid 1-phosphate} (+ \text{ADP})$$

$$\text{D-Galactose} + \text{ATP} \rightarrow \alpha\text{-D-galactose 1-phosphate} (+ \text{ADP})$$

$$\text{L-Arabinose} + \text{ATP} \rightarrow \beta\text{-L-arabinose 1-phosphate} (+ \text{ADP})$$

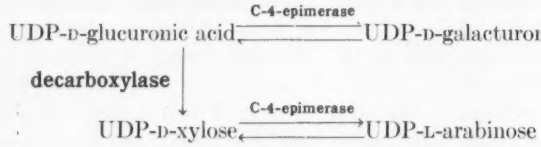
The D-galactose phosphate and L-arabinose phosphate were shown to be phosphorylated on C-1 on the evidence that they are acid labile, and can be converted to the corresponding UDP-sugars when incubated with UTP in the presence of pyrophosphorylase.[59] Similarly, the D-glucuronic acid phosphate is acid labile; but since D-glucuronic acid 6-phosphate would also be expected to be acid labile, no conclusion can be drawn from this fact as to the position of attachment of the phosphate group. However, inasmuch as the D-glucuronic acid phosphate was acted upon by pyrophosphorylase, phosphorylation of this uronic acid must have occurred on C-1. The α-configuration of the D-glucuronic acid 1-phosphate and of the D-galactose 1-phosphate was adduced from the fact that the β-anomers of these sugar phosphates do not react with UTP to form UDP derivatives. The specific rotation of the D-glucuronic acid 1-phosphate (calculated on the basis of the molecular weight of the tripotassium salt) was $[\alpha]_D^{24}$ +56°,[58] which agrees favorably with the value of $[\alpha]_D^{19}$ +53.6° given in the literature for α-D-glucuronic acid 1-phosphate.[60] Similarly, the β-type of linkage of the L-arabinose 1-phosphate was assigned on the basis that this pentose phosphate undergoes a pyrophosphorylase reaction only as the β-anomer.

UDP-D-Glucuronic Acid Decarboxylase.—Physiological experiments by Neish *et al.*[61, 62] and Seegmiller *et al.*[63] with intact plants into which radioactive aldo-

hexoses or D-glucuronic acid were introduced and allowed to metabolize, indicated that the D-xylose and L-arabinose constituent units of pentosans in plants were derived from hexose sugars, presumably through oxidation to uronic acids and subsequent loss of C-6 from the latter.

A similar conclusion was reached by Slater and Beevers[64] from experiments with slices of corn coleoptiles which were allowed to respire in the presence of D-glucuronic acid labeled in C-6 position, and in the presence of randomly labeled D-glucuronic acid. Analysis of the C^{14} labeled CO_2 produced and determination of the radioactivity of the xylan in the tissue suggested the presence of an enzymatic system in the plants that decarboxylates uronic acids to pentoses. It has been postulated that UDP-sugars are involved in the conversions.[61,64]

Neufeld *et al.*[5] demonstrated that cell-free preparations from mung bean seedlings and other plants (asparagus shoots, radish roots and leaves, and spinach leaves) contain an enzyme (decarboxylase) capable of catalyzing the formation of UDP-pentose from UDP-D-glucuronic acid:



In these experiments, particulate preparations from mung bean seedlings were used. They were incubated with UDP-D-glucuronic acid labeled with C^{14} in the D-glucuronic acid moiety. Examination of the reaction mixture after incubation showed that 34 per cent of the UDP-D-glucuronic acid was converted to UDP-pentose (a mixture of UDP-D-xylose and UDP-L-arabinose). Since the preparation also contained UDP-D-galacturonic acid 4-epimerase, UDP-D-galacturonic acid was also formed. It has not yet been determined whether UDP-D-galacturonic acid can also be decarboxylated.

Transglycosylation.—The enzymatic system responsible for sucrose synthesis in plants does not involve a sucrose phosphorylase of the bacterial type that catalyzes a reaction between α-D-glucose 1-phosphate and D-fructose.[65] Leloir *et al.*[10,11] showed that the D-glucose donor for the synthesis of sucrose in plants is UDP-D-glucose, and that plants contain an enzyme, UDP-D-glucose D-fructose transglucosylase, capable of catalyzing the reversible formation of sucrose from UDP-D-glucose and D-fructose. Another enzyme, UDP-D-glucose D-fructose 6-phosphate transglucosylase, present in the same plants, forms sucrose phosphate by a similar reaction when D-fructose 6-phosphate is substituted for D-fructose:

$$\text{UDP-D-glucose} + \text{D-fructose} \rightleftharpoons \text{UDP} + \text{sucrose}$$

$$\text{UDP-D-glucose} + \text{D-fructose 6-phosphate} \rightleftharpoons \text{UDP} + \text{sucrose phosphate}$$

The equilibrium constant K for the reaction for sucrose synthesis from UDP-D-glucose and D-fructose at 37°C and pH 7.4 is approximately 5, which corresponds to a ΔF of about $-1{,}000$ cal. This favorable equilibrium may explain the large accumulation of sucrose in some plants. As in the formation of sucrose, the equilibrium of other transglycosylation reactions involving complex saccharide

formation from UDP sugars would also be expected to be favorable in the direction of synthesis.

In our laboratory, experiments were carried out with mung bean seedlings and other plants, using UDP-D-glucose labeled with C^{14} in the D-glucose moiety as substrate, in an attempt to synthesize cellulose. While our endeavor with regard to formation of cellulose was not fruitful, we have succeeded in synthesizing another polysaccharide (β-1,3-glucan) which is widely distributed in plants.[23]

A solubilized enzyme preparation was obtained from mung bean particles that catalyzed the formation of a water-insoluble radioactive polysaccharide from labeled UDP-D-glucose with the liberation of UDP. The reaction for polymer formation required an activator, which did not appear to be incorporated into the product. D-Glucose and a number of D-glucosides were able to serve as activators.

Partial hydrolysis of the polymer with acid resulted in the formation of a series of oligosaccharides of degree of polymerization from 2 to 8, which were chomatographically identical with those present in a partial hydrolyzate of a β-1,3-linked polysaccharide, laminaran. Chemical degradation of the disaccharide with lead tetraacetate produced arabinose,[66] conclusively proving that the disaccharide is linked between the C-1 and C-3 positions of the two D-glucose units. The oligosaccharides obtained from the synthetic polymer were completely hydrolyzed with emulsin to D-glucose, indicating that the glycosidic linkages are of the β-type. The D-glucose residues in the polysaccharide are therefore joined by β-1,3-glycosidic linkages:

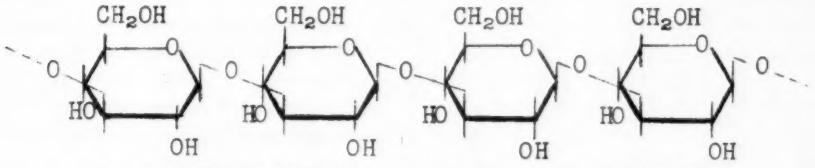


FIG. 2.—Chain of β-1,3-glucopyranose units in synthetic polysaccharide.

Since the D-glucose in the UDP-D-glucose is α-glucosidically bound, the formation of a β-linked polymer must proceed with inversion of configuration, in contrast to the reactions leading to synthesis of glycogen,[20–22] trehalose phosphate,[12] sucrose,[10] and sucrose phosphate,[11] in which the α-linkage is retained. Synthesis of cellulose from UDP-D-glucose by the *Acetobacter xylinum* enzyme[19] and enzymatic synthesis of other complex saccharides[16–18] also involve inversion of the type of linkage.

β-1,3-linked D-glucose polysaccharides are known to be constituents of *Laminaria*, a large family of brown seaweeds (laminaran), and of yeast cell walls (glucan). Recently Aspinall and Kessler[67, 68] showed that the polysaccharide "callose" isolated from the sieve plates of phloem of grape vines is a β-1,3-linked glucan. While it was previously thought that callose was predominantly localized in the phloem tissue of higher plants, it now appears that this polysaccharide is widely distributed in cells of other plant tissues.[69, 70] The polysaccharide is rapidly produced as a response to physiological or mechanical injury to the tissue. It is likely that callose and the β-1,3-glucan synthesized by plant particles from UDP-D-glucose are identical.

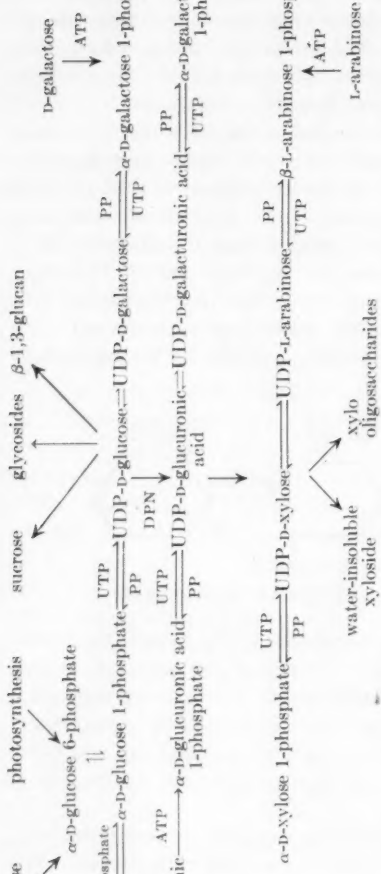


FIG. 3.—Enzymatic formation and various interconversions of sugar nucleotides in plants.

Pentosyl groups, like hexosyl groups, can be transferred to suitable acceptors. Feingold *et al.*[15] presented evidence to the effect that an enzyme preparation from asparagus shoots was capable of catalyzing the transfer of xylosyl units from UDP-D-xylose to β-1,4-linked xylo-oligosaccharides, ranging in degree of polymerization from 2 to 5. This single-step transfer reaction resulted in the production of 1,4-linked oligosaccharides of degree of polymerization ranging from 3 to 6, each containing one more D-xylose residue than the acceptor.

The affinity of the xylosyl transferring enzyme for the xylo-oligosaccharide acceptors proved to be very low, and under the experimental conditions where the ratio of the xylosyl donor to the xylosyl acceptor was 1:120, the addition of more than one xylosyl unit to the oligosaccharide chains could not be observed.

Sugar nucleotides can also serve as glycosyl donors to non-carbohydrate aglycones. Liver microsomes have been shown to transfer the D-glucuronosyl moiety of UDP-D-glucuronic acid to a wide variety of aromatic and aliphatic alcohols, carboxylic acids and amines.[16] Cardini and Yamaha[14] have been able to synthesize phenolic glycosides from UDP-D-glucose in the presence of phenolic acceptors and wheat germ extracts. They were also able to show that in some cases an additional D-glucose unit was transferred from UDP-D-glucose to form a gentiobioside.

When UDP-pentose was incubated in the presence of asparagus particles, a xyloside which was insoluble in water but soluble in hot 80

per cent alcohol was formed. The aglycone moiety (unknown structure) was presumably bound to the particles.[15]

Enzymatic Reactions Leading to Complex Saccharide Formation.—In the photosynthetic reduction of CO_2 by the plant, there is a rapid formation of a number of phosphorylated monosaccharides, among which D-glucose 6-phosphate is found.[71, 72] Phosphoglucomutase converts D-glucose 6-phosphate to D-glucose 1-phosphate, which may then undergo a succession of reactions, resulting in the formation of various sugar nucleotides. Under the influence of the different transglycosylation enzymes in the presence of proper acceptors, the sugar nucleotides serve as donors to form a variety of oligosaccharides, glycosides, and cell-wall polysaccharides.

The following scheme (Fig. 3), showing the formation and the various interconversions of the sugar nucleotides, is based on the enzymatic systems which have been found in mung bean seedlings and in other plants:

It is to be expected that many more reactions involving sugar nucleotides are yet to be discovered. For instance, it is likely that UDP-D-galactose may function as a galactosyl donor in the formation of raffinose and galactan. UDP-D-glucose, a precursor of cellulose in *Acetobacter xylinum*, may also have the same function in the formation of plant cellulose. UDP-D-galacturonic acid may well be a key intermediate in the formation of pectin, while UDP-D-xylose may serve as a xylosyl donor to form the polysaccharide xylan. The formation of araban poses an interesting problem. It seems unlikely that UDP-L-arabinopyranose could be the immediate precursor to araban, in which the L-arabinose residues are usually present in the furanose configuration. A change from a pyranose to a furanose configuration cannot occur unless at some point during the reaction the glycosidic bond combining the L-arabinose to the donor or the enzyme is ruptured. In that event the bond energy would be lost and no polysaccharide could be synthesized. A mechanism for the synthesis of araban from UDP-L-arabinopyranose has been suggested by Roseman.[73] UDP-L-arabinopyranose could react with free L-arabinofuranose to yield a nonreducing disaccharide. By transferring the furanosyl moiety of this disaccharide to an araban primer consisting of L-arabinofuranose residues, the L-arabinofuranose chain would be lengthened, with the regeneration of free L-arabinose.

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*EFFECT OF SOLVENTS AND OF TEMPERATURE ON THE OPTICAL ROTATORY PROPERTIES OF PEPSIN**,†

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In a current project in this laboratory, the chemical and physicochemical properties of pepsin are being investigated in an attempt to define the configuration of the peptide chain or chain segments that determines the biological activity of this enzyme. Inasmuch as a correlation exists between the rotatory properties and the configuration of proteins in solution, the optical rotation of pepsin has been examined in the presence and absence of such reagents as urea, guanidine, and lithium bromide which are known to alter the secondary structure of proteins. Although it has been previously reported by Simpson and Kauzmann and by Jirgensons that the specific rotation of pepsin, $[\alpha]_D$, does not change on short exposure to urea[1] or to guanidine,[2] a certain anomalous behavior, hitherto not yet described for other proteins, has been encountered and will, therefore, be reported here. Moreover, an attempt has been made to relate the optical properties to the enzymic activity of pepsin.

Materials.—The pepsin used in this research was the Worthington crystalline product, lot 623, with a nitrogen content of 14.6$_4$ per cent, the molar extinction coefficient, $\epsilon = 51.3_9 \times 10^3$ at λ 278 mμ and the amino acid composition given elsewhere.[3]

Reagent grade urea and guanidine hydrochloride were recrystallized twice from 60 per cent ethanol and absolute methanol, respectively, whereas the lithium bromide was used without further purification.

Optical Rotation.—The optical rotation was measured in the wave length range

from 350 to 700 mμ with the Rudolph precision ultraviolet polarimeter, model 80, equipped with the photoelectric detector and oscillating polarizer prism. Although most of the determinations were made at 23°C with a Xenon Arc lamp as light source, in some experiments the temperature was varied from 15 to 65°C. Here, water from an external bath was circulated through jacketed cells and served to control the temperature to ±0.05°C. In these measurements the light source was a mercury lamp.

Since the indices of refraction of solvents containing urea, guanidine or lithium bromide differ considerably from water, all specific rotations given in this paper are corrected to the corresponding values in water with the aid of the equation[4]

$$[\alpha]_s = [\alpha] \frac{n^2_{H_2O} + 2}{n_s^2 + 2}$$

In each case the refraction of the solvent was measured with an Abbe refractometer.

Protein Concentration.—Protein concentrations in acetate buffers and lithium bromide were determined from nitrogen analyses performed upon the solutions by the Pregl micro-Kjeldahl method. The factor of 6.83 was used to convert the nitrogen values to dry weight. In the urea- or guanidine- containing solutions the optical density at 278 mμ served to estimate the content of pepsin.

Enzyme Assay.—All solutions were assayed routinely for enzymic activity by the hemoglobin method.[5] In some experiments, the hydrolysis of the synthetic substrate N-acetylphenylalanyl-dl-diiodotyrosine, kindly supplied by Dr. R. Pitt-Rivers, was also tested.

RESULTS

Effect of Urea and Guanidine.—Previous investigations[1, 6] have shown that most globular proteins have a specific rotation, $[\alpha]_D$, in the range of $-30°$ to $-70°$ which on denaturation with urea or guanidine salts becomes more negative by 20° to 60°. Similarly, the optical rotatory dispersion constant, λ_c, is lowered from the range of 230–270 mμ to 212 mμ after contact with these reagents.[7] As a result of these early investigations, and of a study of collagen and gelatin, Cohen suggested in 1955 that the optical rotatory properties could be correlated with the helix content of the protein peptide chain.[8] As an extension of this suggestion Yang and Doty[9] proposed that the optical properties of the two configurations of poly-γ-benzyl-L-glutamate, i.e., the α-helix and the random coil, suffice to explain the characteristics of proteins. Thus a λ_c of 212 mμ is assigned to a protein in randomly coiled form devoid of any helical structure, whereas a higher value of λ_c indicates that a certain fraction of the amino acid residues of the protein is in the helical configuration.

In our investigations of the rotatory properties of pepsin, it immediately became apparent that although the specific rotation at 600 mμ in 0.1 *N* acetate buffer of pH 3.6 to 5.6 is within the range of globular proteins, i.e. $[\alpha]_{600} = -63.5 \pm 0.5°$, the constant $\lambda_c = 216$ mμ is unusually low although agreeing well with that reported by Jirgensons.[10] In all the solvents tested thus far the dispersion follows a one-term Drude equation. Consequently, if the data are expressed as plots of $[\alpha]$ versus $[\alpha] \cdot \lambda^2$, [9] a linear relationship is obtained over the spectral range of 350 to 700 mμ.

If pepsin is dissolved in 8.0 M urea-acetate of pH 5.3_5, the specific rotation and rotatory dispersion is the same as that found in the 0.1 N acetate buffer of the same pH. If, as shown in Table 1, the protein is kept in contact with the solvent at

TABLE 1

SPECIFIC OPTICAL ROTATION AND ROTATORY DISPERSION OF PEPSIN IN UREA-ACETATE AND GUANIDINE HYDROCHLORIDE

Composition of Solvent	pH*	[α] 600 mμ	[α] 400 mμ	λ_c	Relative Specific Activity per Unit Nitrogen† in Per Cent
0.1 N acetate buffer	4.6_4	$63._3$	178	216	100
8.0 M urea-acetate					
0 time	5.3_5	$64._1$	179	218	95
6 hrs., 37°C	5.3_5	$64._9$	175	209	74
24 hrs., 37°C	5.3_5	$64._0$	168	202	10
Urea removed by dialysis against acetate buffer	4.6_4	$60._4$	164	210	66
3.0 M guanidine hydrochloride	3.4	$62._1$	173	217	100
4.0 M guanidine hydrochloride	3.3_8	$59._7$	166	219	100
5.0 M guanidine hydrochloride					
0 time	3.4_1	$60._8$	165	218	93
6 hrs., 23°C	3.4_1	$60._8$	158	200	80
24 hrs., 23°C	3.4_1	$56._1$	149	201	51

* Apparent pH.

† The relative specific activity of a freshly prepared pepsin solution in 0.1 N acetate buffer of pH 4.6 is taken as 100.

37°C for an extended period, $[\alpha]_{600}$ remains unchanged but λ_c decreases from 218 mμ to 202 mμ during a period of 24 hours. This change of λ_c is not due to a helix-coil transition as one would expect if a great number of hydrogen bonds were broken. Rather the presence of urea enhances the rate of autodigestion which is accompanied by an apparent inactivation of the enzyme and the formation of dialyzable material.[11] Since the optical rotatory dispersion constant of low molecular weight peptides is lower than that of proteins, the λ_c of 202 mμ, given in Table 1, reflects the contribution of these peptides and is superimposed on the value of λ_c of the residual protein. On removal, by dialysis, of the low molecular weight material, the rotatory dispersion constant of the non-dialyzable fraction increases to 210 mμ, a value similar to that of the starting material. Furthermore, the residual non-dialyzable fraction has a high specific activity per unit of nitrogen. Although the value of λ_c given above is still somewhat below that of pepsin it is due to the residual 34 per cent of inactive but nondialyzable components, a conclusion confirmed by electrophoretic fractionation of the nondialyzable material. It is thus clear that the changes described here do not originate from an alteration of the secondary structure but are probably a result of the autolysis process. A behavior similar to that described above is encountered if pepsin is dissolved in guanidine hydrochloride. As may be seen in Table 1, the presence of 3.0 M guanidine hydrochloride does not affect the optical parameters, $[\alpha]$ and λ_c, nor the enzymic activity. In 5.0 M guanidine hydrochloride, the specific rotation, $[\alpha]_{600}$, however, increases somewhat and λ_c decreases from 216 mμ to 200 mμ during a 30-minute exposure to the reagent. If the rotatory properties of pepsin in this solvent are followed over a period of 24 hours, the specific rotation at all wave lengths becomes less negative without the value of λ_c being affected. It has also been recognized recently that the presence of guanidine salts in the pH range of 3.0 to 5.0 enhances the rate of

autodigestion of pepsin.[12] At 3.0 *M* guanidine hydrochloride at 25°C, autolysis is slow but occurs at an appreciable rate if the concentration of the reagent or the temperature is raised.

From the results to date, one can conclude that pepsin solutions at 20° to 25°C do not contain appreciable amounts of the protein in the α-helix form. Invariably if a lowering of λ_c occurs in the presence of the so-called hydrogen bond breaking reagents, it coincides with the formation of low-molecular weight peptides that results from the autodigestion of the protein.

Effect of Lithium Bromide.—Harrington and Schellman have recently investigated the effect of concentrated lithium bromide on the optical rotatory properties of several proteins. In view of the fact that this reagent lowers the activity of water, these investigators suggest that in certain instances at least, lithium bromide may strengthen hydrogen bonds, i.e., favor helix formation. Such an effect should cause changes opposite to those usually observed with urea or guanidine. The specific rotation, $[\alpha]$, should be less negative whereas λ_c should increase if compared with the values found in buffer solutions. Changes of this type were actually observed by Harrington and Schellman[4, 13] when ribonuclease, clupein, silk fibroin, and serum albumin were examined in solutions of lithium bromide.

Inspection of Table 2 reveals that addition of a given concentration of lithium

TABLE 2

SPECIFIC OPTICAL ROTATION AND ROTATORY DISPERSION OF PEPSIN IN LITHIUM BROMIDE AND AFTER REMOVAL OF LITHIUM BROMIDE BY DIALYSIS AGAINST 0.1 *N* ACETATE BUFFER, pH 4.6

Composition of Solvent	pH*	$[\alpha]$ 600 mμ	$[\alpha]$ 400 mμ	λ_c	Relative Specific Activity per Unit Nitrogen in Per Cent
1.0 *M* LiBr-acetate	4.4$_8$	62.$_6$	168	208	100
2.0 *M* LiBr-acetate	4.3$_2$	61.$_3$	173	207	66
LiBr removed†	4.6$_4$	65.$_4$	184	218	100
3.0 *M* LiBr-acetate	4.0$_8$	61.$_6$	169	205	40
LiBr removed†	4.6$_4$	66.$_3$	185	218	100
4.0 *M* LiBr-acetate	3.9$_6$	56.$_7$	160	218	24
LiBr removed†	4.6$_4$	66.$_4$	161	215	100
5.0 *M* LiBr-acetate	3.8$_8$	54.$_8$	154	219	18
LiBr removed†	4.6$_4$	67.$_4$	187	213	89

* Apparent pH.
† LiBr removed by dialysis against 0.1 *N* acetate buffer of pH 4.6.

bromide reduces the activity of the enzyme by an amount proportional to the salt concentration and that no further change occurs. The specific rotation, $[\alpha]_{600}$, is not altered appreciably up to 3.0 *M* lithium bromide. However, λ_c decreases from 216 mμ to 205 mμ. In contrast to urea and guanidine, no increase of trichloroacetic acid soluble material occurs. On increasing the concentration of lithium bromide to 5.0 *M*, the highest concentration at which pepsin remains soluble in this reagent, the specific rotation is less negative and λ_c increases from 205 to 218 mμ. In every instance removal of lithium bromide by dialysis restores the enzymic activity, measured either with hemoglobin or the dipeptide, N-acetyl-phenylalanyl-dl-diiodotyrosine as substrates. Moreover, the values of $[\alpha]$ and λ_c approach those found for pepsin in acetate buffer in the pH range of 3.6 to 5.6.

Although these results are in apparent contrast with those of Harrington and

Schellman, and can be taken as an indication that no appreciable helix formation takes place in the case of pepsin, it should be pointed out that a phenomenon similar to the one described here has recently been noticed by Harrington with collagen and gelatin.[14]

Effect of Temperature.—Since at 25°C the optical rotatory properties of pepsin do not undergo changes if "hydrogen-bond breaking" or "hydrogen-bond strengthening" reagents are added to a pepsin solution, the effect of temperature on the optical rotatory properties has been investigated. A point of interest immediately emerges from Table 3. In 0.1 *N* sodium acetate buffer of pH 4.6, the specific rotation $[\alpha]_{546}$ and $[\alpha]_{365.5}$, respectively, increase slightly and λ_c changes from 216 to 226 mμ, if the temperature is raised from 40 to 60°C. Throughout all these measurements, the enzyme remains fully active and no increase in nonprotein material as tested by the solubility of the protein in hot 10 per cent trichloroacetic acid occurs. Inactivation of the enzyme starts at temperatures above 60°C and the change of $[\alpha]$ and λ_c precedes somewhat the loss of biological activity.

Similarly, if urea or guanidine hydrochloride are added, λ_c also increases with temperature. However, a marked loss of peptic activity has already occurred at 45°C. In no case could formation of low-molecular weight material be detected, thus excluding the influence of autodigestion on either the activity or on the altering of the optical properties of the protein as is the case below 40°C. Moreover, it should be pointed out that $d[\alpha]/dT$ is negative in the acetate buffer, but positive in urea and guanidine. Here again the behavior of pepsin is in direct contrast to that of other proteins, e.g., β-lactoglobulin.[13]

The data in Table 3 show the temperature dependence of the optical rotatory

TABLE 3

SPECIFIC OPTICAL ROTATION AND ROTATORY DISPERSION OF PEPSIN IN ACETATE, 8.0 *M* UREA-ACETATE, 5.0 *M* GUANIDINE HYDROCHLORIDE AND LITHIUM BROMIDE-ACETATE AS FUNCTION OF TEMPERATURE

Composition of Solvent	pH*	Temperature in °C	[α] 546 mμ	[α] 365.8 mμ	λ_c	Relative Specific Activity per Unit Nitrogen in Per Cent
0.1 *N* acetate buffer	4.3	23°	78.6	232	218	100
		50°	77.6	232	227	99
		55°	75.9	228	228	103
		60°	75.1	221	226	100
8.0 *M* urea-acetate	5.3	23°	77.7	229	218	95
		50°	78.3	237	223	11
		55°	79.7	241	229	5
		60°	80.2	241	229	1
		65°	79.3	240	230	0
5.0 *M* guanidine hydrochloride	3.5	23°	76.4	224	218	100
		45°	75.5	222	220	5
		50°	77.8	226	220	...
		55°	77.4	226	226	4
2.5 *M* LiBr-acetate	4.1	20°	75.6	223	205	100
		40°	76.1	225	219	103
		45°	77.7	226	220	104
		50°				106
		55°		not measured		93
5.0 *M* LiBr-acetate	3.6$_5$	25°	69.2	204	218	88
		37°	69.0	202	223	73
		45°	65.4	198	230	33
		50°	63.3	198	235	2

* Apparent pH.

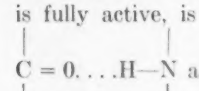
properties of pepsin in the presence of 2.5 *M* and 5.0 *M* lithium bromide. In 2.5 *M* lithium bromide $d[\alpha]/dT$ is positive and λ_c increases from 205 mμ to 220 mμ if the temperature is raised to 50°C. The enzymic activity is not affected. In 5.0 *M* lithium bromide, on the other hand, $d[\alpha]/dT$ is negative. If the pepsin solution is kept at 37°C for 15 minutes appreciable inactivation can be detected, both if tested in the presence or absence of the reagent.

It can be concluded from these results that an increase of the rotatory dispersion constant, λ_c, from 216 to 226–230 mμ, invariably precedes or accompanies the loss of the biological activity of the enzyme.

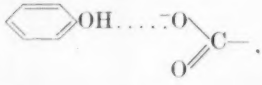
Discussion.—In the present investigation it has been shown that the enzymically active form of pepsin in solution is characterized by certain optical parameters, $[\alpha]$ and λ_c. In all solvents thus far examined, an increase of λ_c coincides with an irreversible loss of activity. On the other hand, a lowering of λ_c, in most instances is indicative of the presence of low-molecular weight material that arises from autodigestion of the enzyme. In view of the peculiar properties of pepsin, the results of the optical rotatory measurements will be discussed in terms of the amino acid composition of the protein.

Pepsin, with a molecular weight of 35,000 is a protein with a single peptide chain[15] which consists of 343 amino acid residues and is cross linked by three disulfide bonds. From the low intrinsic viscosity of the protein, $\eta = 3.0_9$ gm. ml, it follows that the peptide chain is compactly folded. Moreover, the protein is unusual in that it has 71 acidic and only 4 basic amino acids and possesses an exceptionally high content of proline and of serine. Thus, it is quite likely that the low value of $\lambda_c = 216$ mμ is a reflection of the large amounts of glutamic, aspartic acid, and serine.[3]

The fact that on short exposure of pepsin to urea or guanidine in the pH range of 3.0 to 5.0 at 25°C, the optical rotatory properties remain unchanged and the enzyme is fully active, is of special interest. Although hydrogen bonds of the type



and those involving the phenolic hydroxyls of tyrosine and the carboxylate ions of the acidic amino acid residues, i.e.,



undoubtedly exist in pepsin[16] they must be relatively unimportant in maintaining the configuration necessary for the enzymic activity of this protein. Moreover, if the 18 proline residues are distributed statistically along the peptide chain only short helical segments could exist since the presence of the pyrollidine ring in the peptide chain prevents helix formation. Hence, one can assume that pepsin has a "random coil" configuration. Moreover, in view of the fact that hydrogen bond breaking reagents are ineffective in altering the secondary structure, other bonds such as hydrophobic bonds may play a role in stabilizing the molecule, a view further supported by the fact that 63 per cent of the amino acid residues are of nonpolar nature.‡

An alternative explanation for the "apparent lack" of an extensive helical con-

figuration follows from the fact the pepsin has 41 aspartic acid residues and 44 serines. Blout and Karlson[17] and Fassman[18] reported recently that the optical rotatory dispersion of β-benzyl-L-aspartate and of poly-L-serine differs markedly from that of poly-L-glutamate and other polyamino acids. The anomalous rotatory behavior of pepsin could, therefore, be explained if sequences of the type -Asp·Asp·Asp-. . .or -Ser·Ser·Ser- would be present in this protein and would counteract the contribution of the other amino acids to the "right-handed helix."

Although the decrease of λ_c at low concentrations of lithium bromide distinguishes pepsin from the behavior of the globular proteins, such as ribonuclease, a similar change of the dispersion constant has recently been reported by Harrington and Sela[19] if poly-L-proline or proline-glycine copolymers are dissolved in lithium bromide. These investigators proposed that a trans-cis isomerization of the type described by Kurtz, Berger, and Katchalski[20] is mediated by lithium bromide. Although the occurrence of trans-cis isomerization has not yet been demonstrated in proteins, it is not unlikely that it may exist if the proline residues were present in sequences of the type -Pro·Pro-, -Pro·Pro·Gly- or -Gly·Pro·Pro- and may play a considerable role in biological systems.

A further point of interest is that unlike β-lactoglobulin, insulin and clupein[13] the optical rotatory dispersion constant, λ_c, of pepsin increases if the temperature is raised above 45°C and that this change coincides with or precedes the inactivation of the protein. This phenomenon may be due to the fact that certain amino acid residues, e.g., proline, lock the peptide chain into a certain configuration which confers considerable stability upon the protein. At higher temperatures, however, transition to a less stable configuration takes place. It is clear that in the case described here the chain segment necessary for the biological activity of the enzyme has been affected.

From the results presented in this communication, it is apparent that the application of the optical rotatory dispersion to the study of the secondary structure of proteins can be rendered even more powerful and unambiguous if supplemented by the use of other chemical and physicochemical methods.

Summary.—The effect of solvents and temperature on the optical rotatory dispersion of pepsin has been investigated. Urea and guanidine salts do not affect the specific optical rotation, $[\alpha]$, at 600 mμ. When a lowering of the rotatory dispersion constant λ_c from 216 to 200 mμ occurs, it has been shown to be a consequence of autodigestion of the enzyme. At temperatures above 45°C an increase of λ_c from 216 to 226–230 mμ takes place which coincides with a loss of the enzymic activity of pepsin. It may be concluded that the helical content of pepsin is negligible.

My sincere thanks go to Dr. Ephraim Katchalski for having introduced me to this type of work and to Dr. William F. Harrington of the National Institutes of Health for helpful discussion and for the hospitality of his laboratory where some of the measurements were carried out. The author is indebted to Mrs. Inese Leimanis for her able assistance in the activity measurements.

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‡ Pepsin is insoluble in hydrophobic bond-breaking reagents. Although it is possible to dissolve the protein in dichloroacetic acid, an irreversible loss of activity occurs instantaneously; likewise exposure to 2-chloroethanol inactivates pepsin whether the assay is conducted in the presence or absence of the reagent.

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*UNITY IN THE SPECIFICITY OF ENZYME AND ANTIBODY INDUCTION BY THE SAME DETERMINANT GROUPS**

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Introduction.—For many years it has been hypothesized that induced enzyme formation and antibody production are closely related processes. As pointed out by Pollock,[1] this proposal was made by Dienert in 1900, who described the "adaptation" of yeast to galactose, at the time when immunochemical phenomena were first coming under intensive investigation. A wealth of experimental evidence has since accumulated, showing that both of these protein-forming systems utilize amino acids rather than larger peptide units as precursors, that they are under strict genetic control, and that the site of guidance for their specificity may be a template of ribonucleoprotein. The extensive literature which has led to these conclusions has been reviewed by Borsook,[2] Spiegelman,[3] Haurowitz,[4] and Burnet.[5]

One major difference in the detailed mechanisms involved in induced enzyme *versus* antibody protein formation lies in the well-known fact that the former process is initiated by an inducer (determinant) of low molecular weight and simple structure, whereas the latter system requires an antigen having an obligatory molecular

weight of many thousands. Yet, the classical studies of Landsteiner[6] have demonstrated that the specificity of the antibody can be directed against simple determinant groupings (haptens) which may be attached to the carrier antigen. If one makes the assumption that the microbial induction of specific enzymes and the mammalian formation of specific antibodies differ essentially only in the ability of the microorganisms to attach the inducer either to pre-existing or to newly-formed high molecular weight "carrier," around which the appropriate enzyme synthesizing apparatus can fold, it seems not unlikely that the specificity engendered by a common determinant grouping in each of these systems would be similar.

An ideal basis for the comparison of specificity in these two protein synthesizing systems presents itself in the careful delineation of the structural requirements for substrates, for inducers, and for induction-inhibition of β-galactosidase in *E. coli* by Monod and co-workers,[7] and in *B. megaterium* by Landman;[8] and in the study of Gross and Tatum[9] on the induction and inhibition of protocatechuic acid oxidase in *Neurospora*. By coupling galactose in β-glycosidic linkage to bovine serum albumin, and by conjugation of protocatechuic acid to the same protein fraction through its carboxyl group, we have prepared synthetic antigens presumably corresponding to possible inducer-template complexes. Rabbit antisera to these antigens were tested with the appropriate enzyme inducer-substrates, inducer-nonsubstrates and inhibitors, for the ability of these compounds to act as hapten inhibitors. It has thus been possible to demonstrate a parallelism between the stereochemical and conformational requirements of enzyme induction and those for the antibody protein forming system in a direct experimental manner.

Materials and Methods.—Antigens: The synthesis of β-galactosyl phenylazo proteins for use as antigens has previously been described by Goebel and Avery.[10] *p*-Aminophenyl-β-D-galactoside, $[a]_D^{25} = -40.3°$, was prepared essentially by their procedure, and was coupled by diazotization to bovine serum albumin (BSA, Armour fraction V) by the procedure by Westphal and Feier.[11] Analysis of the purified antigen by the anthrone method showed that 14 sugar residues were attached per mole (assumed M. W. 70,000) of BSA.

Protocatechuic acid, vanillic acid, and 2,4-dihydroxybenzoic acid, respectively, were coupled to BSA by the mixed anhydride method previously applied by Erlanger *et al.*[12] to the preparation of steroid-protein conjugates. In a typical experiment, 2.3 gm. (0.03 mMole) of BSA were dissolved in a mixture of 40 ml. of water and 50 ml. of tetrahydrofuran, and the pH was brought to 10.6 by adding 20 ml. of 0.1 *N* NaOH. The mixed carbonic anhydride was prepared by treatment of 0.237 gm. protocatechuic acid (1.5 mMole) in 15 ml. cold tetrahydrofuran with 0.36 ml. tri-*n*-butylamine (1.5 mMole) and then with 0.2 ml. isobutylchlorocarbonate (1.5 mMole). After 15 minutes in the ice-bath, an additional 0.7 ml. of tri-*n*-butyl-amine was added, and the entire contents of this flask were added at once to the chilled protein solution. The reaction mixture was stirred in the cold for 20 minutes, and then for an additional 20 minutes at room temperature. Following 24-hour dialysis against cold running tap water, the solution containing the protein conjugate was chilled and treated with dilute HCl, whereupon the conjugate precipitated. The precipitate was centrifuged, suspended in water, and dissolved by the addition of $NaHCO_3$ to pH 6.8. After further dialysis for several days, protocatechuic acid-bovine serum albumin conjugate (PCA-BSA) was isolated by

lyophilization. Analysis of the aroyl-protein conjugates, by acid hydrolysis and spectrophotometric determination of the resultant ether-extractable hapten, demonstrated that an average of 20 aromatic residues were attached per mole (assumed M. W. 70,000) of BSA.

Immunochemical Procedures: Antisera were obtained from rabbits immunized with alum-precipitates of each of the conjugated haptens.[13] All antisera were absorbed with BSA before use in order to remove any antibody which was specific for BSA and not for the introduced haptenic group. Quantitative antibody determinations followed the procedures described by Kabat and Mayer.[13] The specific precipitates were analyzed with the Folin-Ciocalteu[14] reagent, and the hapten inhibition technique was employed qualitatively[6] and quantitatively[15] to study the specificity of the antisera. The various haptens were incubated with the antiserum prior to the addition of the homologous conjugated antigen. The effectiveness of each hapten in inhibiting precipitation was calculated from a comparison of the precipitate formed in the presence of the hapten to the amount which was formed in control tubes containing only the antibody and antigen.

Haptens: Most of the polyhydroxy aromatic acids and analogs were commercially available and were recrystallized to their proper physical constants before use. We should like to thank Dr. S. R. Gross for a generous sample of protocatechuic acid (PCA). We are indebted to Drs. Spiegelman, Cohn, Halvorson, Isbell, and Wolfrom for many of the thiogalactosides and sugar derivatives used in these experiments. Other galactosides and analogs were synthesized by previously published methods.

Results.—PCA-BSA, antiPCA-BSA System: In Table I are presented data for

TABLE 1

COMPARISON OF SUBSTITUTED AROMATIC COMPOUNDS AS HAPTEN-INHIBITORS WITH THEIR FUNCTION IN INDUCTION OF PROTOCATECHUIC ACID OXIDASE IN *Neurospora**

Compound	Conc. μg.	Hapten Inhibition	Inducer in *Neurospora*[9]
Protocatechuic acid	100	+	+
Vanillic acid	100	+	+
m-Hydroxy benzoic acid	100	+	+
p-Hydroxy benzoic acid	100	+	+
Gallic acid	100	+	+
2,4-Dihydroxy benzoic acid	1000	−	−
2,6-Dihydroxy benzoic acid	1000	−	−
Anisic acid	1000	−	−
p-Amino benzoic acid	1000	−	±
Isovanillin	1000	−	?
Vanillin	1000	−	±
Gentisic acid	1000	−	−
Salicylic acid	1000	+	−

* Inhibition determined qualitatively by visual comparison of precipitate formed in presence and absence of hapten.

hapten inhibitions, compared visually with control tubes, for this antibody-antigen reaction. It can be seen that there is a good correlation between the ability of compounds to act as hapten inhibitors, and the relative effectiveness of the same compounds in the induction of protocatechuic acid oxidase in *Neurospora*. Especially noteworthy was the finding that 2,4-dihydroxybenzoic acid, a noninducer but a competitive inhibitor of the enzyme itself, is not a good hapten inhibitor, whereas vanillic acid, a good inducer for the mold enzyme synthesis, is a powerful hapten

inhibitor in the immunochemical test. From these data, it would appear to a first approximation, that the specificity of hapten inhibition follows closely the pattern of specificity for enzyme induction in *Neurospora*. In contrast, salicylic acid is an apparent exception, since it acts as a hapten inhibitor, but cannot induce the synthesis of the *Neurospora* enzyme. Separate tests demonstrated that salicylic, acid, at these concentrations, inhibited even the nonrelated BSA-anti-BSA reaction. This nonspecific binding is probably due to attachment of the salicylate anion to the cationic centers of the protein, as was shown by Lindenbaum and Schubert.[16] In fact, the problem of nonspecific binding of the various aromatic polyhydroxycarboxylic acids proved to be so great that it was not possible to make any quantitative comparisons on the basis of the relative immunochemical effectiveness of these compounds in inhibiting aroylprotein, antiaroyl-protein precipitations. However, these qualitative preliminary results obtained with the protocatechuic acid conjugates indicated that the experimental approach was feasible, and our attention was next directed toward the β-galactosidase system, wherein nonspecific interferences due to solvolysis and to ionization would be minimal.

β-gal-BSA, anti-β-gal-BSA System: A comparison of the specificity of induction of β-galactosidase in *E. coli* and in *B. megaterium* with that of hapten inhibition in the anti-β-phenylazo-galactoside system is given in Table 2. It can be seen that simple carbohydrates such as D-glucose, D-mannose, D-xylose, D-arabinose, D-galacturonic acid, 2-deoxy-D-galactose, and L-galactose, which are inert in the bacterial inducible systems, likewise demonstrate a lack of activity in this immunochemical test. The same is true for the disaccharides D-maltose, D-cellobiose, and the oligosaccharide D-raffinose. Furthermore, ethyl-β-D-galacto-furanoside was not a hapten-inhibitor while all β-D-galacto-pyranosides listed were potent hapten-inhibitors. This result is entirely consistent with the finding by Zarnitz[17] that crystalline β-galactosidase from *E. coli* ML 309 hydrolyzes β-D-galacto-pyranosides, but does not attack phenyl-β-D-galacto-furanoside.

L-arabinose, which has the same stereochemical configuration as does D-galactose, acts as a weak hapten-inhibitor in the antigen-antibody reaction. This compound induces β-galactosidase synthesis in *B. megaterium* but not in *E. coli*, but does not combine with the enzyme. Melibiose and lactose, which on a molar basis are weak enzyme inducers in *E. coli*, are also weak hapten inhibitors, as is neolactose which is a noninducer substrate. Phenyl-α-D-galactoside, also a weak hapten-inhibitor, was the only compound tested which combines with the antibody, but which does not react in the induced enzyme system. An optical rotation determination showed that the preparation used contained about 4 per cent of the β-isomer which may account for a good part of its hapten-inhibiting activity.

The correspondence between the induced enzyme and antibody forming systems in so far as structural specificity for inversion at the carbon atom #4, and for α- and β- anomerism is borne out by the results of the experiments run with α-D-galactosides and β-D-glucosides, all of which are devoid of inhibitory activity. *o*-Nitrophenyl-β-D-galactoside, which on a quantitative basis is one of the best inducers and substrates for β-galactosidase, is correspondingly one of the best hapten inhibitors, giving *ca.* 50 per cent inhibition at 0.25 μMolar concentration. The enzyme substrate 6-bromo-2-naphthyl-β-D-galactoside is furthermore one of the most effective of the galactosides tested in the immunochemical system.

TABLE 2

COMPARISON OF SUGARS AND GLYCOSIDES AS HAPTEN-INHIBITORS WITH THEIR FUNCTION IN INDUCED ENZYME FORMATION IN *E. coli* AND *B. megaterium*

Potent Hapten Inhibitors

Compound	Conc. for Approx. 50% Inhibition (μM)	Activity in Induced *E. coli* and/or *B. megaterium* Systems [1, 7, 8, 18]		
		Inducer	Enzyme Affinity	Permease Affinity
p-Aminophenyl-β-D-galactoside	0.11	−*	−	−
2-Naphthyl-β-D-galactoside	0.13	+	+	−
6-Bromo-2-naphthyl-β-D-galactoside	0.13	−	+	−
o-Nitrophenyl-β-D-galactoside	0.25	+	+	−
p-Aminophenyl-β-D-thiogalactoside	0.35	+	+	−
Phenyl-β-D-galactoside	0.39	+	+	−
iso-Propyl-β-D-thiogalactoside	1.1	+	−	+
Methyl-β-D-thiogalactoside	1.2	+	+	+
Methyl-β-D-galactoside	2.3	+	+	−
Galactosido-β-D-thiogalactoside	8.4	−	+	+
D-Galactose	27.8	+	+	−
Phenyl-β-D-thiogalactoside	0.37	+	+	+

Weak Hapten Inhibitors

	Conc. for Approx. 20% Inhibition (μM)			
Phenyl-α-D-galactoside	2–3.9	0	−	−
Neolactose	3.4	0	+	−
Lactose	8.8	+	+	+
Melibiose	29.2	+	+	−
L(+) Arabinose	33.3	+	0	−

Inactive Sugars (No Significant Inhibition)

	Highest Conc. Tested (μM)			
Methyl-α-D-galactoside	3.6	0	0	−
p-Nitrophenyl-β-D-glucoside	5.0	−	−	−
p-Nitrophenyl-α-D-glucoside	5.0	−	−	−
D-Galacturonic acid	5.2	0	0	−
L-Galactose	5.6	−	−	−
D-Raffinose	6.0	0	0	−
2-Deoxy-D-galactose	6.1	0	0	−
L-Arabitol	6.6	−	−	−
Phenyl-β-D-glucoside	7.4	−	−	−
D-Maltose	14.6	0	0	−
Cellobiose	14.6	0	0	−
Ethyl-β-D-galacto-furanoside	24.2	−	−	−
D-Mannose	27.8	0	0	−
D-Glucose	27.8	0	0	−
D-Xylose	33.3	0	0	−
D-Arabinose	33.3	0	0	−

* (−) Indicates no data found in the literature.

Another interesting series of correlations can be made by considering the experimental results obtained in both protein-forming systems with respect to the thiogalactosides. Again stereo-specificity with respect to inversion at carbon atom #4 holds true, since the β-thioglucosides are inactive as hapten inhibitors. Phenyl-β-D-thiogalactoside, which does not induce enzyme production in *E. coli*, but which inhibits the fully formed enzyme from this microorganism, is known to act as an inducer in *B. megaterium*. On a molar basis it is one of the most efficient hapten inhibitors of the analogous antibody-antigen reaction. The closeness of fit in this particular biological immunochemical test would require, if our original premise be correct, that the compound act somewhere in the induction process leading to β-galactosidase. The fact that it is active at the enzyme-forming site in one case,

and at the enzyme active surface in the other, indicates that this generalization has validity. Finally, it should be pointed out that the thio analogs methyl-β-D-thiogalactoside and *p*-aminophenyl-β-D-thiogalactoside, which are nonsubstrate inducers for β-galactosidase of *B. megaterium*, also fit well into the surface profile of the antibody.

Recently, another biological phenomenon has been described which has a bearing on this problem. This is the "permease" enzyme system, at the surface of the *E. coli* cell. It has been shown, for example,[18] that the analog β-thiodigalactoside is an inhibitor not only for the induced enzyme itself, but that this compound is very effective in preventing induction at the "permease" site. It was not surprising, therefore, that this substance when tested with the mammalian antiserum to the β-galactosido-protein also demonstrated a strong specific bonding to the active site.

Discussion.—With rare exceptions, compounds capable of inducing β-galactosidase, acting as substrates or inhibitors for the complete enzyme, or acting as inducers or inhibitors of "permease" in *E. coli* and in *B. megaterium*, also specifically combined with the antibody to the β-galactosido-phenylazo-protein produced in the rabbit. A similar correspondence was found between the induced protocatechuic acid oxidase of *Neurospora* and the rabbit antibody to the protocatechuoyl-protein. In spite of the probable extensive difference in the over-all structure of the induced enzyme and of the specific antibody, the data strongly suggest that the surface profile of the active site on a protein, which is specific for a given low molecular weight determinant group, will be similar in any organism genetically endowed to form it, and that specificity resides primarily in the molecular conformation of the determinant.

The specificity for β-galactosides and for aromatic hydroxy carboxylic acids was artificially introduced into the antibodies by the classical technique of forming a stable chemical bond between the hapten and the antigen. It is believed that a corresponding bond, with the same orientation and with a reasonable half-life, must be formed between the inducer and the initial receptor for the inducer in microbial systems. Thus, in the case of β-galactosidase induction, this bonding could occur by transglycosidation, with stereospecificity still maintained in a chemically active inducer-receptor linkage. Likewise, aromatic carboxylic acids or amino acids acting as inducers may go through an initial activation at their carboxyl groups, *via* adenylic anhydrides, which could then transfer the specific informative group to the receptor, thus creating a relatively stable link. The interesting results obtained with induced penicillinase enzyme from *B. cereus*[1] (where induced cells in a medium freed of the inducer continue to make enzyme) can be considered in light of this hypothesis as being due to the formation of a strong undissociable bond by acylation of the receptor macromolecule through opening of the β-lactam ring of the inducer itself.

At the outset of this investigation, it was thought that it might be possible to correlate the specificity of the antibody either with the specificity of the enzyme-forming system, or with the specificity of the induced enzyme. In the protocatechuic acid system, the qualitative findings indicated more of a parallelism to the induction process. Consideration of the anti-β-D-galactoside system—for which the data are far more reliable and for which there already exist in the literature

results obtained for several microbial inductions—indicates that the apparent distinction between specificity for induction and specificity for structural requirements of inhibitors or substrates of the induced enzyme, is fast disappearing. As previously mentioned, pheny-β-D-thiogalactoside which does not induce enzyme production, but which inhibits the fully-formed enzyme in *E. coli*, has been shown to act as an inducer in *B. megaterium*. Where the data are available, it can be seen that all compounds which are potent hapten inhibitors have more than one activity with respect to the induced systems (i.e., enzyme affinity, induction, and permease affinity). Only two of the good hapten-inhibitors, 6-bromo-2-naphthyl-β-D-galactoside (a subtrate) and *p*-aminophenyl-β-D-galactoside (no published data), have not been studied in more than one system. It would appear, from our data and our original premise, that these two substances should be active in the induction process leading to β-galactosidase.

There appears to be little doubt that the site of induction is probably located in the cellular organization elsewhere than at the enzyme level. Moreover, although the size of the antibody combining site is probably larger[15] than the induction site, the induction site might have a profile analogous to the terminal portion of the combining site of the antibodies produced against the haptens (inducers). It might therefore be possible to use antiserum to an inducer as a reagent for determining the actual site of induction. Since both the induction site and the antibody when mixed together should then compete for the inducer (hapten), the effect of such competition might be quantitatively determined by the methods herein described, employing various subcellular fractions of an inducible microorganism.

Summary.—Antigens containing protocatechuic acid or the β-galactosido- groups coupled to bovine serum albumin have been synthesized. Antibodies to these antigens have been obtained whose specificity toward various hapten analogs parallels the specificities previously found for these compounds acting as inducers, substrates, and inhibitors in induced microbial systems. These results suggest that directed protein synthesis, stimulated by a specific determinant group, takes place by a similar mechanism in all biological systems.

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*AN EARLY PHASE IN THE BACTERICIDAL ACTION OF 5-FLUOROURACIL ON E. COLI K_{12}: OSMOTIC IMBALANCE**

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Since the discovery of the carcinostatic action of 5-fluorouracil (FU)[1] the mechanism of inhibition by this antimetabolite has been studied in tumor cells,[2] in viral[3] and in microbial systems.[4, 5]

It has been shown that the interference of FU with cellular syntheses is complex: the free base FU inhibits uridine synthesis by blocking the enzyme uridine phosphorylase[6] and after being assimilated into various nucleotide derivatives by the cell, FU inhibits the synthesis of thymine by blocking the methylation of deoxyuridylic acid.[5] The ribonucleotide derivative of FU is incorporated into the RNA of tumor cells, bacteria, and tobacco mosaic virus, substituting for uracil in the polymers.[2] Disturbances of protein synthesis in FU treated bacteria have also been reported.[7]

Cohen *et al.*[5] in their extensive studies on the toxicity of FU in *E. coli B* strains have summarized the action of FU as affecting both uracil and thymine metabolism; effects related to uracil requirements inhibited growth but not survival, while effects related to the creation of thymine deficiency were markedly bactericidal.

During studies of the action of FU on *E. coli* K-12 we have observed a new feature of FU inhibition, namely, the existence of an early phase during which the restoration of the bacterial cell to viability can be achieved in a hyperosmotic *milieu*.

When *E. coli* K-12 cells in exponential growth phase in synthetic medium are exposed to various concentrations of FU (2 μg per ml up to 1 mg per ml gave qualitatively the same results) for several generation times and are plated at intervals on ordinary nutrient agar plates for the determination of colony forming viable cells, the number of "dead" cells in the population increases at a high rate. However, if identical aliquots of such drug-treated organisms are plated on nutrient agar plates into which high concentrations of neutral salts, sucrose, or other solutes are incorporated, the apparent survival rate is completely altered. For example, if, after 60 minutes of exposure to the drug the bacterial culture is plated on ordinary nutrient agar plates, over 95 per cent of the cells fail to give rise to colonies; however, plated on hyperosmotic nutrient agar plates close to 95 per cent of the original cells become viable colony formers. In Figure 1 the difference in viable colony forming cell counts on ordinary nutrient agar plates‡ and on nutrient agar plates supplemented with sucrose§ is indicated.

E. coli K-12 cells which had not been exposed to FU always yielded the same number of colonies on the normal or on any of the supplemented plates used. The

restoration of the otherwise "dead" organisms seems to be a function of the osmotic strength of the medium rather than some specific effect of the supplements. Such a conclusion is suggested by the following facts: Potassium, sodium or ammonium chlorides, nitrates or sulfates as well as sucrose, maltose, mannose, and mannitol were equally effective. Iso-osmotic concentrations of the various salts gave the same recoveries (at around 0.2 *M* ionic concentrations). This was also true in the case of nonelectrolyte supplements (Table 1).

TABLE 1

Supplement in the Nutrient Agar Plate	Concentration, *M*	Viable Cells per Plate
None	....	18
Sucrose	0.68	217
Mannitol	0.68	220
Mannose	0.68	225
Maltose	0.68	210

An interesting feature of the phenomenon is that the number of colonies recovered increased proportionally with the supplement concentration. (Beyond certain maximum concentrations however, the number of colonies diminished due to specific toxicities of the individual supplement used.)

In the experiment illustrated in Figure 2, exponentially growing cultures of *E. coli* K-12 (1×10^8 cells per ml) were exposed to 1 mg per ml of FU for 90 minutes and then plated—after appropriate dilutions—on nutrient agar plates containing increasing concentrations of sucrose. It is apparent that there is a nearly linear relationship between the log of surviving cells and the sucrose concentration, up to 20 per cent of sucrose.

In the same experiment aliquots were also plated on nutrient agar plates supplemented with various concentrations of both thymine and uracil with and without salt in the agar. The results summarized in Table 2 show that high concentrations of uracil or thymine cannot substitute for the osmotic requirement for recovery.

TABLE 2

Supplements Incorporated in the Nutrient Agar Plates, μg/ml		Viable Cells per Plate
0.5% NaCl	Uracil (100) Thymine (100)	157
Incorporated	Uracil (1000) Thymine (1000)	153
No NaCl	Uracil (100) Thymine (100)	14
	Uracil (1000) Thymine (1000)	10
	No supplement	24

The restoration of otherwise "dead" cells to viability by hyperosmotic *milieu* is reminiscent of the properties of protoplasts.[8] We have therefore searched for evidences of protoplast formation. Examination in a phase contrast microscope of cultures of *E. coli* K-12 exposed to FU for one hour revealed no protoplasts at all. However, the cells appeared somewhat thickened. On longer incubations with the

drug (2–3 hours), the cells became elongated structures having 3 to 5 times the length of normal log phase *E. coli* K-12 cells. This growth in size is probably responsible for the slowly but continuously increasing turbidity of FU treated cell suspensions which continues even on very long incubations. Paralleling the elongation of the cells, which has been described in bacteria under a number of other conditions,[9] a dense granulation also developed in the FU treated cells.

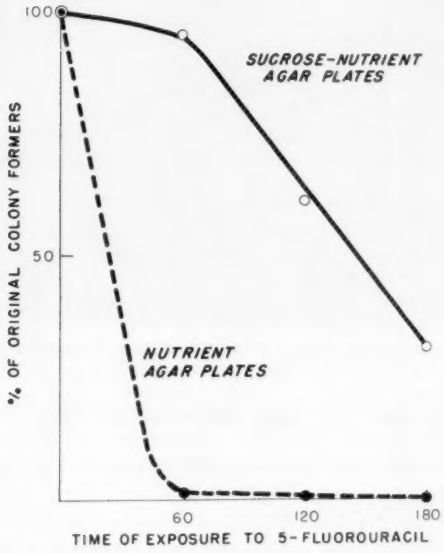

FIG. 1.—The viability of *E. coli* K_{12} after exposure to 1 mg/ml of 5-fluorouracil, on ordinary nutrient agar plates and on nutrient agar plates supplemented with 20 per cent sucrose.

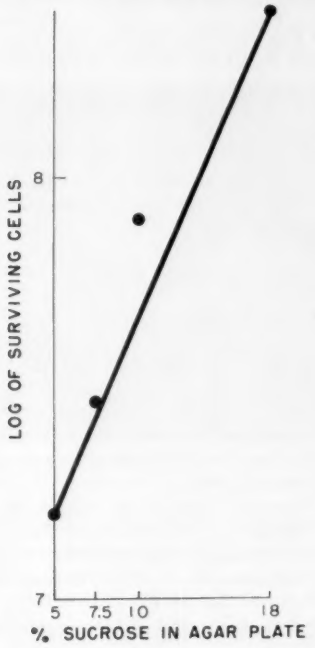

FIG. 2.—The effect of increasing concentrations of sucrose in the nutrient agar plates on the survival of *E. coli* K_{12} after exposure for 90 minutes to 1 mg/ml of 5-fluorouracil.

The phenomenon described above and called here for brevity "osmotic restoration" was not observed in cells of *E. coli* B exposed to FU under similar conditions. The phenomenon may be unique to strains of *E. coli* K-12. That the "osmotic restoration" may be related to some interference of FU in the uracil metabolism of the cell rather than in thymine metabolism is indicated by the following experiments.

To an aliquot of *E. coli* K-12 1 mg per ml of FU and an equimolar concentration of uracil, to another 1 mg of FU and an equimolar concentration of thymine were added simultaneously. After aerobic incubation for one hour aliquots were plated from each culture on normal and on salt-supplemented agar plates. It was found that uracil provided full protection against the osmotic imbalance, i.e., 100 per cent of the original population formed viable colonies on both kinds of agar plates. However, in the cultures with thymine as the additional adjuvant only 40 per cent

of the original population gave rise to viable colonies on normal agar plates, but 100 per cent were successful colony formers on supplemented agar plates.

The mechanism of "osmotic restoration" is obscure. It may be the result of an early injury of the cell wall synthesizing mechanism leading to a transitional imbalance between the synthesis of cell wall and protoplasmic material;‖ or, perhaps, it may be a reflection of the interference of FU with some other phase of pyrimidine metabolism.

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‡ Prolonged incubation of normal nutrient agar plates—up to 3 days—instead of the usual 16 hours did not increase the number of colonies.

§ On plates containing high concentrations of sucrose or salt the size of the colonies was appreciably smaller than on normal nutrient agar plates after identical times of incubation. When single "dwarf" colonies were restreaked on normal nutrient agar plates they gave rise to normal sized colonies and the number of such colonies on normal and on supplemented plates was the same.

‖ The role of uridine coenzymes in bacterial cell wall synthesis has been demonstrated.[10]

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*A DEMONSTRATION OF THE RECOVERY OF THE CROWN-GALL TUMOR CELL WITH THE USE OF COMPLEX TUMORS OF SINGLE-CELL ORIGIN**,†*

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Introduction.—It is a generally accepted belief that the cellular alteration leading to malignant tumor cell types represents a permanent change. Cells that possess low grades of neoplastic change may and frequently do become further altered in the direction of greater malignancy. Once this state has been attained, however, such cells have not been observed to turn back *en masse* toward the benign or normal states. Studies on the origin of a tumor cell (except the virus-induced tumors) have been complicated in the past by an inability to accomplish a controlled recovery of

such cells and thus permit an experimental analysis of the several hypotheses that have been advanced to account for the continued abnormal and autonomous growth of a tumor cell.

The typical crown-gall tumor cell of plants, like malignant animal cells, appears to be a permanently altered cell that reproduces true to type and against the growth of which there is no control mechanism in the host. This cell type is characterized both in the host and in culture by a capacity for continued rapid proliferation, by a limited capacity for differentiation, and by the lack of a capacity to organize tissues and organs (Fig. 1, A). Since tumor cells of this type have not in the more than 10

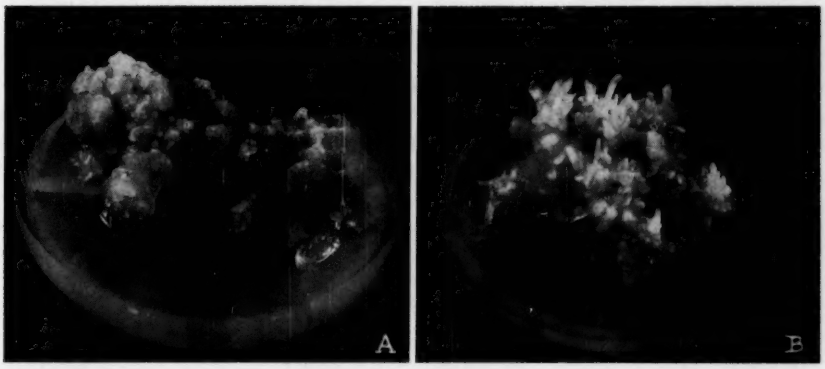

FIG. 1.—A. Typical tobacco crown-gall tumor tissue of the unorganized type. B. Crown-gall teratoma tissue of tobacco derived from a single cell. (Photographs by J. A. Carlile.)

years that they have been kept under observation shown the slightest tendency to become less autonomous, they have generally been considered to be permanently altered cells and hence unsuited for studies dealing with the recovery of a tumor cell.

A second morphologically quite distinct type of crown-gall tumor may be produced in certain plant species the cells of which possess highly developed regenerative capacities. Tumors of this type possess, when they develop in a host, and retain indefinitely when planted in culture, a pronounced capacity to organize highly abnormal leaves and buds (Fig. 1, B). The pluripotent cells of which these teratomata are composed appear to retain, despite their alteration to tumor cells, highly developed regenerative capacities.

The complex tumors, like those of the unorganized type, are transplantable. The growth pattern of the resulting tumor depends, however, upon the position that the implant occupies in a host.[1] Furthermore, cells isolated from teratomata grow indefinitely, as do cells obtained from an unorganized tumor, on a simple inorganic salts-sucrose-containing culture medium that does not support the continued growth of normal cells of the type from which the tumor cells were derived. Since the complex tumors possessed a capacity to organize abnormal tumor buds, they were found to be admirably suited and were used for studies on the origin of the crown-gall tumor cell.[2] An attempt was made in earlier investigations to distinguish between somatic mutation at the nuclear gene level and the presence of a self-perpetuating cytoplasmic entity that had assumed control of the cells and was responsible for the

continued abnormal proliferation of the affected cells. It is well known in biology that certain self-duplicating cytoplasmic entities can be eliminated from cells under conditions that favor the increased multiplication of those cells in relation to the multiplication of the self-duplicating factor. Since normal meristematic cells of a rapidly growing bud divide with far greater frequency than do most crown-gall tumor cells, it was hypothesized that, if the tumor buds found to develop from the teratomata could be forced into very rapid growth, recovery of the tumor cells might be accomplished provided the factor responsible for the continued abnormal proliferation of the tumor cells was subject to the effects of dilution in very rapidly dividing cells. The results of that study showed that when tumor shoots derived from tumor buds were forced into rapid growth by a series of graftings to healthy plants, they gradually recovered and became normal in every respect. These results suggested that the cellular alteration in crown gall did not involve a somatic mutation at the nuclear gene level since heritable changes of that type are not generally believed to be lost as a result of rapid growth. They suggested, rather, that some autonomous or partially autonomous entity, which is subject to the effects of dilution in very rapidly dividing cells, is present in and is responsible for the continuity of the tumorous properties from one cell generation to the next. This interpretation of the observed results was subsequently questioned by certain investigators because it was felt that, on the basis of the evidence presented, the possibility that the teratoma tissue was composed of a stable mixture of normal cells and tumor cells had not been eliminated.[3-5] This question could be resolved unequivocally only if clones of teratoma tissue of single-cell origin were used in the investigations. Although methods for the culture of single plant cells had not been developed at the time that the original studies were reported, such methods are now available.[6] This has permitted a re-examination of the question of the recovery of the crown-gall tumor cell. It is with that phase of the crown-gall tumor problem that the present study is concerned.

Experimental Methods.—The clone of tobacco crown-gall teratoma tissue used in this study has now been maintained in culture for somewhat more than 5 years. Throughout this period the tissue has been characterized by a pronounced capacity to organize numerous abnormal-appearing leaves and buds and to grow continuously at a moderately fast rate on a modified White's culture medium containing 1 per cent agar. The teratoma tissue was maintained in this laboratory on a modified medium containing four times the concentration of the basic salts recommended by White.[7]

The single cells used in this study were obtained as follows. The liquid culture medium consisted of White's basic medium supplemented with a 10 per cent extract of juice obtained from rapidly growing *Vinca rosea* crown-gall tumors grown in tissue culture. The tumor extract was found to favor growth of the tumor cells. This medium was further supplemented with naphthalene acetic acid at a final concentration of 0.01 mg/liter. Fifty cc of the medium was placed in each of several 250-ml Erlenmeyer flasks. The flasks were stoppered and sterilized in an autoclave.

Teratomata that had grown on a solid medium for 5–6 weeks were cut into small fragments several millimeters in diameter and a large number of such fragments were placed in the medium described above. The flasks were shaken rapidly on a re-

ciprocal shaker for a period of about 5 weeks. Microscopic examination at the end of that period showed numerous single cells and clumps of cells to be present in the medium (Fig. 2, A, B). A suspension of such cells was either transferred to fresh medium of the type described above and again shaken or it was spread evenly on an agar surface in a Petri dish for isolation of single cells.

The single cells were isolated and cultivated according to the method described by Muir, Hildebrandt, and Riker.[6] The "host" or stock tissue upon which the filter paper (Reeve Angel, crepe surface no. 202) was placed was normal *Nicotiana glutinosa* tissue that had been carried continuously in culture for 10 years on White's basic medium supplemented with 0.5 mg/liter naphthalene acetic acid and fortified with 1 per cent agar. Normal *N. glutinosa* tissue had been found to be a favorable nurse tissue for tobacco teratoma cells. In these studies a cube of the normal tissue was cut in such a way that each side had a diameter of about 7 mm. A square piece of sterile filter paper 8–9 mm in diameter was placed upon the freshly cut upper surface of the tissue fragment. The tissue containing the paper was planted

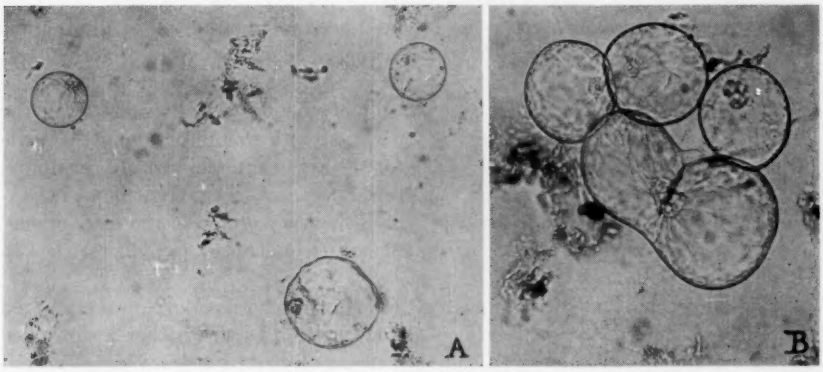

FIG. 2.—A. Single cells of the type from which the teratoma tissue shown in Fig. 1, B was derived. B. Small cluster of cells. Note that one of the cells is undergoing division. (Photographs by J. A. Carlile.)

in 125-ml Erlenmeyer flasks containing White's solid medium supplemented with naphthalene acetic acid at a concentration of 0.05 mg/liter. The tissue was allowed to remain in contact with the agar for 3 days, after which time the tissue and filter paper were removed as a unit and placed in a sterile Petri dish.

Single teratoma cells that had been planted on an agar medium in a Petri dish were isolated under a dissecting microscope with the aid of a platinum microspatula that was flattened and pointed at the tip. The single cells were planted on the filter paper. The tissue containing the filter paper was then again placed on the agar medium from which it had been removed. The entire procedure was carried out as rapidly as possible to prevent drying of the single cell.

The grafting of tissues of single-cell origin and of shoots that developed from such tissues was carried out according to methods previously described.[2,8]

Experimental Results.—Of a total of 267 single teratoma cells isolated and planted on filter paper, 12 or somewhat more than 4 per cent grew. Of these, 1 became contaminated during the course of incubation, 4 multiplied and reached diameters up to

0.3 mm but failed to continue growth, while 7 reached diameters up to 5 mm and were planted on a modified White's basic medium where they continued to grow well independently. The tissue of each of the 7 clones grew in an unorganized manner and showed no macroscopic evidence of organization at the time that they were transplanted to the basic medium from the filter paper. However, after the tissues of 6 of the 7 clones had reached diameters of between 0.7 and 1 cm, they organized morphologically abnormal structures at their surfaces. The tissue of each of the 7 clones was subdivided into 4 pieces and these were again planted on modified White's basic medium. The tissues derived from 6 of the clones grew in a manner comparable in every respect to the teratoma tissue from which the single cells were derived. Tissues of the seventh clone failed to organize during two successive passages in culture. During the third passage, morphologically highly abnormal leaves and buds developed from several of the tissues in this clone. The capacity of tissues of all clones derived from single cells to organize persisted through subsequent subdivisions and plantings on modified White's basic medium.

The results of this study indicate that the capacity of teratoma tissue of single-cell origin to organize is a reflection of the inherent potentialities of pluripotent tumor cells and is not the result, as has been suggested, of a mixture of normal and tumor cells. The possibility exists, of course, that, as the progeny of the single cells grow, some recover, perhaps as a result of unequal cell divisions, from the effects of the tumor-inducing principle associated with this disease and that it is those recovered cells that are responsible for the organizational capacity exhibited by the teratoma tissue. Although this possibility cannot at present be tested experimentally, there is every indication that, if recovery occurs in the manner suggested above, it does not happen with sufficient frequency to account for the observed results.

The finding that clones of teratoma tissue of single-cell origin developed organized structures permitted a re-examination of the question as to whether a controlled recovery of crown-gall tumor cells could be accomplished by forcing organized but morphologically abnormal tumor buds into rapid growth at the shoot apex by means of a series of graftings to healthy tobacco plants. In these studies teratoma tissues of single-cell origin and about 5–7 mm thick were grafted to cut stem ends of tobacco plants from which the axillary buds had been removed. In those instances in which the tumor grafts were successful, they developed into overgrowths of considerable size. The surfaces of these growths were covered with organized structures many of which were highly abnormal in appearance. An examination of free-hand sections of such grafted tumor tissue showed that the organized structures arose from the tumor tissue at the periphery of the expanding tumor mass and not from tissues of the normal plant upon which the graft was made.

The procedures used in an attempt to effect the recovery of crown-gall teratoma cells were similar to those previously described.[2] Shoots that developed from the tumor buds present in the teratoma grafts were removed when they were between 3 and 5 cm long and were grafted to the cut stem ends of healthy tobacco plants. Some of these shoots were very abnormal in appearance and grew slowly and very abnormally in length and in thickness. After they had reached an appropriate length, the tips were again removed and grafted to healthy tobacco plants. These commonly grew more rapidly and became more normal in appearance. The tips

of such shoots were again removed and grafted to healthy plants. They developed rapidly and appeared normal in every respect, ultimately flowering and setting seed. Such seed was sown and the resulting plants were found to be normal *Nicotiana tabacum* plants (Fig. 3). In these instances recovery appeared to have been complete. It was a gradual process that progressed in the direction of the normal as the affected shoots were forced into very rapid growth as a result of a series of graftings to healthy plants.

FIG. 3.—A normal tobacco plant of the type obtained by forcing morphologically abnormal shoots derived from teratoma tissue of single-cell origin into very rapid growth by means of a series of graftings to healthy plants. A complete recovery from the tumorous state has resulted from this procedure. (Photograph by J. A. Carlile.)

In certain other instances, recovery was achieved more quickly. When, for example, some of the more normal-appearing shoots that developed from the grafted teratomata of single-cell origin were grafted to the cut stem tips of healthy tobacco plants, they grew rapidly after an initial delay and a complete recovery was achieved in a single grafting. The cells of such recovered scions failed to grow on a basic culture medium that supported the continued rapid growth of crown-gall tumor cells. The cells of the recovered plants were also found to be again susceptible to transformation by the tumor-inducing principle associated with this disease.

Discussion.—Clones of complex crown-gall tumors derived from single cells were used in investigations described above. The results of these studies indicate that when pluripotent plant cells are transformed into crown-gall tumor cells, they may retain indefinitely a capacity to organize morphologically highly abnormal leaves and buds. The organizational capacity exhibited by such cells is an expression of the inherent potentialities of the pluripotent tumor cells and does not in itself appear to affect the recovery of those cells. Only when organized structures such as those found in tumor shoots derived from the morphologically abnormal tumor buds were forced to divide very rapidly at the shoot apex, did they recover and ultimately become

normal in every respect. These findings make somatic mutation at the nuclear gene level appear unlikely as a possible explanation for the nature of the cellular alteration in crown gall. They suggest, instead, that some as yet uncharacterized cytoplasmic entity is responsible for the cellular changes that underlie the tumorous state in the crown-gall disease. This does not, of course, preclude the possibility that such postulated cytoplasmic changes may be more or less under control of the nuclear genes. These findings, which are very suggestive of some that have been encountered in microbial genetics, may be interpreted by assuming that the factor responsible for the continued abnormal growth of the crown-gall tumor cell is an autonomous or partially autonomous entity that is subject to the effects of dilution in very rapidly dividing cells.[9] While such a particle hypothesis appears to explain quite satisfactorily the experimental findings reported here, so little is in fact known about the mechanics of cell division, differentiation and organization that other interpretations, such as, for example, Delbrück's[10] suggestion involving steady state chemistry in which alternative chains of metabolic reactions are assumed to compete with one another, may apply.

This study demonstrates unequivocally, furthermore, that the progeny of a single somatic parenchymatous cell of tobacco may possess all of the potentialities necessary to reconstitute an entire tobacco plant.

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† Some preliminary studies on this problem were carried out in the laboratory of Professor F. C. Steward while the author was Visiting Professor in the Botany Department at Cornell University, Ithaca, New York, in 1957. The author is indebted to Professor Steward and to Miss Kathryn Mears for their kind assistance and interest in this investigation.

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EQUILIBRIUM SEDIMENTATION IN A DENSITY GRADIENT OF MATERIALS HAVING A CONTINUOUS DISTRIBUTION OF EFFECTIVE DENSITIES

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Recent work[1, 2] has shown that equilibrium sedimentation in a density gradient will separate solutes whose effective densities[3] differ very slightly and will provide information from which molecular weights can be computed. This is of particular interest in the study of deoxyribonucleic acid (DNA), as Meselson, Stahl and Vinograd[1] have shown, not only because one can study the replication of DNA[2] but also because there is disagreement between the molecular weights measured for DNA samples by other methods.[4] Since the method is known to be sensitive to small differences in effective density (θ), one would like to know the effects of a continuous distribution in θ. On the one hand it would be useful to measure such distributions, and on the other hand it is important to know how a distribution of θ affects the calculation of molecular weights.

For a single solute, the shape of the curve of concentration versus distance (the "band") is Gaussian,[1] to a first approximation, and the band width is inversely proportional to the square root of the molecular weight, M. Thus a linear plot of $\log c$ versus $(r - r_0)^2$ (where c is concentration, r is distance from the center of rotation, and r_0 marks the center of the band) has been used[1, 2] to test for homogeneity, and M has been found from the slope of this graph. It will be shown below that if the material has a Gaussian distribution of effective densities *the band will still be Gaussian in shape* and the width of the band will be markedly increased by narrow distributions of θ. Thus a Gaussian shape for the band is a necessary but not sufficient condition for homogeneity in θ, and the presence of a Gaussian distribution of θ will cause M to be seriously underestimated.

When the material being studied contains several fractions, we may express the total concentration C as the sum of the individual concentrations (which are given on a weight per volume scale)

$$C = \sum_i c_i \tag{1}$$

and denote the weight fraction of each by

$$w_i = c_i^\circ / C^\circ \tag{2}$$

where c_i° is the initial concentration of species i. Combination of equations (1) and (2) gives

$$C_r = C^\circ \sum_i w_i (c_i / c_i^\circ)_r \tag{3}$$

If the material has a continuous distribution, $g(\theta)$ of species each with an effective density θ the following equation takes the place of equation (3).

$$C_r = C^\circ \int_0^\infty g(\theta)\ (c/c^\circ)_{r,\theta}\, d\theta \tag{4}$$

It has been shown by Meselson *et al.*[1] that under certain conditions discussed in the following section, c is given by

$$\ln\ (c/c_{r_0}) = -(r - r_0)^2/2\sigma^2 + (0)\ (r - r_0)^3 \tag{5}$$

$$\sigma^2 = RT/M\bar{v}\omega^2 r_0 \left(\frac{d\rho}{dr}\right)_{r_0} \tag{5a}$$

$$\theta \equiv \rho_{r_0} = 1/\bar{v} \tag{5b}$$

where $(0)\ (r - r_0)^3$ means that (minor) terms of order higher than $(r - r_0)^2$ are omitted. Here R is the gas constant, T the absolute temperature, ρ the density of the solution, ω the angular velocity of the rotor, and $\bar{v}$ the partial specific volume (cc/gm) of the solute. In the next section it will be shown that equation (5) retains this form, but that a different significance must be given to σ^2 and θ, when the effects of preferential interaction are considered. The relation of c_{r_0} to the initial concentration c° depends on the type of cell used. In general

$$\int_a^b Ac\, dr = c^\circ \int_a^b A\, dr \tag{6}$$

where A is the cross-sectional area of the cell and the limits b, a mark the ends of the column of solution. For a rectangular cell, use of equation (6) gives

$$c_{r_0} = c^\circ(b - a)/\sigma\sqrt{2\pi} \tag{7a}$$

and for a sector-shaped cell the result is

$$c_{r_0} = c^\circ(b^2 - a^2)/2r_0\sigma\sqrt{2\pi}. \tag{7b}$$

When the distribution of effective densities is Gaussian

$$g(\theta) = \frac{1}{\gamma\sqrt{2\pi}}\, e^{-(\theta - \theta_m)^2/2\gamma^2} \tag{8}$$

equation (4) can be integrated readily by completing the square, provided that σ is treated as a constant and ρ is expressed by[5]

$$\rho = \rho_m + \left(\frac{d\rho}{dr}\right)_{r_m} (r - r_m) + (0)\ (r - r_m)^2 \tag{9}$$

In equation (8), θ_m is the mean of the distribution of θ and γ is the standard deviation in equation (9), r_m is the value of r at which $\rho = \theta_m$. For a rectangular cell the result of integration is

$$C = \frac{C^0(b - a)}{\varphi\sqrt{2\pi}}\, e^{-(r - r_m)^2/2\varphi^2} \tag{10}$$

$$\varphi^2 = \sigma^2 + \left(\gamma/\frac{d\rho}{dr}\right)^2 \tag{10a}$$

Thus a material with a Gaussian $g(\theta)$ will form a Gaussian band. As equation (10a) shows, the width of the band will be markedly increased by small values of γ. For example, a value for γ of 0.003 gm cm^{-3} would double φ^2 for each of the

DNA bands shown in Figure 2 of the article by Meselson and Stahl[2] and cause M to be underestimated by a factor of 2. Since $\theta_m = 1.7$ gm cm^{-3} for this case, such a value for γ would represent a standard deviation equal only to 0.2 per cent of the mean. Narrow distributions of θ such as this could arise both from chemical and from configurational differences among the molecules. Heating DNA was found[2] to change θ_m by 5 times this amount ($\Delta\theta_m = 0.016$ gm cm^{-3}). Differences in base composition produce sufficient variation in θ that DNA samples from different organisms actually produce separate bands in a cesium chloride gradient.[6] It would not be surprising if differences in base composition among the DNA molecules of one organism were sufficient to cause broadening of this band.

There is a close analogy between the equations given above and the ones which describe boundary spreading in electrophoresis for a material with a distribution of mobilities. In the latter case, the concentration gradient curve is Gaussian for a single solute and also for a material with a Gaussian distribution of mobilities,[7] provided the diffusion coefficient is the same for all species and the field strength and mobilities can be assumed constant. One can measure $g(\theta)$ when the band is not Gaussian in shape by the equation of Brown and Cann.[8] Their equation, which gives the distribution of mobilities in terms of moments of the experimental curve, can be adapted to the sedimentation case simply by changing the notation. However, one must have an independent estimate of σ^2 (equations 5a, 17a). One would like to measure $g(\theta)$ without knowing σ^2 and, by analogy with the measurement of mobility distributions by an extrapolation to infinite time,[9, 10] one might hope to do this by varying ω. However this is not possible, as equations 5a and 10a show: the relative contributions to φ^2 of σ^2 and $\left(\gamma/\frac{d\rho}{dr}\right)^2$ do not vary with ω, since $d\rho/dr$ is proportional to ω^2.

Alternatively one might hope that, when the material is examined both in a rectangular cell and in a sector cell, the band shapes would be sufficiently different to detect the heterogeneity in effective density.[11] The result of integrating equation (4) for the case of a sector-shaped cell is

$$C = \frac{C^0(b^2 - a^2)}{2r_m\varphi\sqrt{2\pi}}\, e^{-(r-r_m)^2/2\varphi^2}\left\{1 + \frac{\epsilon\sigma^2}{r_m} - \epsilon(r - r_m) + \epsilon^2(r - r_m)^2 + \ldots\right\} \tag{11}$$

$$\epsilon = 1/r_m\left[1 + \left(\sigma\frac{d\rho}{dr}/\gamma\right)^2\right] \tag{11a}$$

The additional terms of equation (11) are only slightly larger than the ones omitted from equation (5), and both are outside present experimental error when light-absorption optics are used. Consequently, examining a material in these two types of cells offers little promise of detecting heterogeneity in θ.

A method of detecting heterogeneity in θ which does appear promising (Meselson *et al.*,[1] footnote 1) is the use of a partition cell to isolate material on either side of the band center, followed by rebanding of the isolated materials. The new bands should differ from that of the starting material both in shape and position. One might be able to detect differences in position by using schlieren optics to find the

positions of the bands, and by examining both materials simultaneously in a twin cell experiment.

At this point we will consider certain of the assumptions made in deriving equation (5), in order to see what factors might cause the band for a single solute with the properties of DNA to deviate from Gaussian shape. First, there is the question of the interaction of the solute with itself: equation (5) applies to the limiting case in which the concentration of solute approaches zero. Since one can study DNA at very low concentrations (*ca.* 0.001 gm/100 ml) with light absorption optics, the assumption seems reasonable. Secondly, there is the question of charge effects, caused by dissociation of the polyelectrolyte into ions. These have been considered by Meselson *et al.*[1] and by Yeandle,[12] and the conclusion has been reached that the shape of the band remains Gaussian. Thirdly, there is the question of preferential interaction of the solute with one of the components of the mixed solvent, a situation which is known to affect markedly the light scattering behavior of such a system.[13] An equation has already been given for the effect of this on the position of the center of the band.[14]

Consider a system of three nonionizing components: a mixed solvent whose components are labeled 0, 1, and a macromolecular solute labeled 2. In order to obtain a simple equation which applies rigorously to compressible systems, it is convenient to use a molal, or weight per weight, concentration scale. Equation (43) of Williams *et al.*[14] provides a convenient and rigorous starting equation.

$$\lim_{W_2 \to 0} \frac{1}{W_2} \frac{dW_2}{dr} = \frac{\omega^2 r M_2 (1 - \bar{v}_2 \rho)}{RT} \left\{ 1 + \frac{\Gamma'(1 - \bar{v}_1 \rho)}{(1 - \bar{v}_2 \rho)} \right\} \tag{12}$$

$$\Gamma' = -(m_1 \beta_{21} M_1 / M_2)/(1 + m_1 \beta_{11}) \tag{12a}$$

$$\beta_{ik} = \left(\frac{\partial \ln \gamma_i}{\partial m_k} \right)_{T, P, m} \tag{12b}$$

Here W is the number of grams and m is the number of moles per 1000 gm of component 0, and γ_i is the activity coefficient of component i on the molal scale. If we again define r_0 to be the position of the maximum concentration

$$\frac{dW_2}{dr} = 0, \qquad r = r_0 \tag{13a}$$

and define θ, as before by the value of ρ at which $r = r_0$, we have[14]

$$\theta \equiv \rho_{r_0} = \left[\frac{1 + \Gamma'}{\bar{v}_2 + \Gamma' \bar{v}_1} \right]_{r_0} \tag{13b}$$

When the variables on the right-hand side of equation (12) are expressed in series form as functions of $(r - r_0)$

$$r = r_0 \left[1 + \left(\frac{r - r_0}{r_0} \right) \right] \tag{14a}$$

$$X = X_{r_0} + \left(\frac{dX}{dr} \right)_{r_0} (r - r_0) + (0)\,(r - r_0)^2 \tag{14b}$$

$$\lim_{W_2 \to 0} \left(\frac{dX}{dr}\right)_{r_0} = \left[\left(\frac{\partial X}{\partial m_1}\right)_P \frac{dm_1}{dr} + \left(\frac{\partial X}{\partial P}\right)_{m_1} \frac{dP}{dr}\right]_{r_0} \tag{14c}$$

where X = either ρ, $\bar{v}_2$, $\bar{v}_1$, or Γ', and then these expressions are substituted into equation (12), the result is

$$\lim_{W_2 \to 0} \frac{1}{W_2} \frac{dW_2}{dr} = -\frac{(r - r_0)}{\sigma_W{}^2} + (0)\,(r - r_0)^2 \tag{15}$$

$$\sigma_W{}^2 = RT/(M_2\omega^2 r_0) \left\{\bar{v}_2 \frac{d\rho}{dr} + \rho \frac{d\bar{v}_2}{dr} - \frac{d\Gamma'}{dr}(1 - \bar{v}_1\rho) + \Gamma'\left(\bar{v}_1 \frac{d\rho}{dr} + \rho \frac{d\bar{v}_1}{dr}\right)\right\}_{r_0} \tag{15a}$$

Integration with respect to $(r - r_0)$ gives, as before, a Gaussian curve.

$$\lim_{W_2 \to 0} \ln\,[W_2/(W_2)_{r_0}] = -\frac{(r - r_0)^2}{2\sigma_W{}^2} + (0)\,(r - r_0)^3 \tag{16}$$

In order to obtain an equation on the c scale, for comparison with equation (5); we consider the case in which $d\bar{v}_1/dr$ and $d\bar{v}_2/dr$ are negligible and λ', the quantity corresponding to Γ', is a function only of the concentrations. Then, following the same procedure outlined in equations 12–16, one obtains the result

$$\lim_{c_2 \to 0} \ln\,[c_2/(c_2)_{r_0}] = -\frac{(r - r_0)^2}{2\sigma^2} + (0)\,(r - r_0)^3 \tag{17}$$

$$\sigma^2 = RT/(M_2\omega^2 r_0)\left[(\bar{v}_2 + \lambda'\bar{v}_1)\frac{d\rho}{dr} - (1 - \bar{v}_1\rho)\left(\frac{\partial\lambda'}{\partial c_1}\right)_P \frac{dc_1}{dr}\right]_{r_0} \tag{17a}$$

$$\lambda' = -\left(\frac{c_1 M_1}{M_2}\right)\left(\frac{\partial \ln y_2}{\partial c_1}\right)_P \Big/ \left[1 + c_1\left(\frac{\partial \ln y_1}{\partial c_1}\right)_P\right] \tag{17b}$$

$$\theta \equiv \rho_{r_0} = \left[\frac{1 + \lambda'}{\bar{v}_2 + \lambda'\bar{v}_1}\right]_{r_0} \tag{17c}$$

If the partial volumes $\bar{v}_2$ and $\bar{v}_1$ are known, one can find λ' by measuring θ and using equation (17c). Then one can find M_2 from σ^2, by means of equation (17a), if $(\partial\lambda'/\partial c_1)$ is approximated by (λ'/c_1). This procedure will probably be accurate enough for measurements made with light absorption optics. More rigorous procedures could be devised by using equations (15) and (16).

In summary, if the macromolecular material examined by this method has a Gaussian distribution of effective densities it will form a Gaussian band and thus appear to be homogeneous. However, the band width will be increased and the apparent molecular weight will be less than the true molecular weight, often by a large amount. When the effects of preferential interaction of the solute with one component of the solvent are considered, one finds that the band for a single solute is still Gaussian but that its position and width are altered. One can take account of this, at least approximately, if the partial volumes are known.

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AGGREGATION OF DYES BOUND TO POLYANIONS

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Acridine orange (AO) is one of a number of dyestuffs which aggregate in aqueous solution. It is thought that these flat, aromatic dye molecules aggregate by stacking on top of one another, and are held together by London dispersion forces between their π-electron systems. The argument for aggregation in the case of AO rests upon a quantitative analysis by Zanker[1] of the variation in the dye spectrum with concentration and temperature. As the dye concentration is increased, the absorption band (at 492 mμ) of the monomer falls and is replaced by a new band (at 464 mμ) due to dimers. With further increases in concentration this band shifts further toward shorter wave lengths, corresponding to the formation of higher aggregates. Zanker showed that these changes could be quantitatively expressed in terms of an association equilibrium constant, corresponding to a free energy decrease in forming a dimer of 5.7 kcal/mole.

When AO is used to stain certain polyanionic tissue elements or is mixed with

the polyanions in dilute aqueous solution, the color either of the monomer or of the aggregates may appear, depending upon the particular polymer involved and the relative amounts of polymer and dye. The appearance of more than one color in tissue stained by a single dye was termed metachromasy by Ehrlich. Michaelis[2] proposed that metachromatic dyes are able to aggregate when bound to the surfaces of polyanions just as they do in solution. He explained the color changes observed when the relative amount of polymer is decreased as being equivalent to those accompanying the concentrating of dye in solution: with a large excess of polymer the dye spectrum contains only the monomer, or α, band. As the amount of polymer is decreased and the dye molecules are crowded onto a smaller surface area, the α band falls and the dimer, or β, band appears. In some polymers, if the

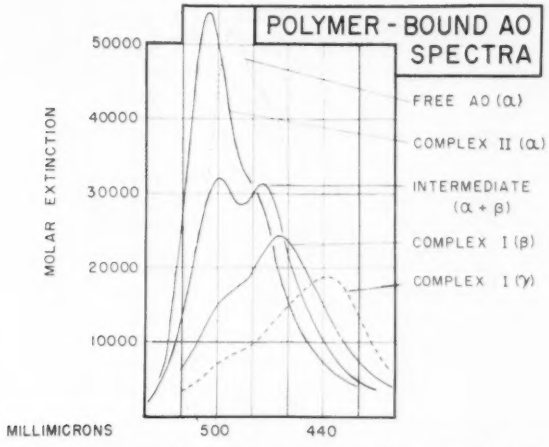

FIG. 1.—Spectra of AO in dilute aqueous solution (2×10^{-5} M) in the absence of polymer (Free AO) and bound to polyanions. The bound AO spectra vary with the type of polymer, when each binding site is occupied (Complex I—β and γ types), and with the number of excess binding sites: Complex II—α type, with large excess; intermediate—$\alpha + \beta$ type, with smaller excesses.

concentration of polymer is reduced still further a higher aggregate, or γ, band appears. Subsequent workers[3,4] have shown that the α band continues to fall and the β and γ bands continue to rise with decreasing polymer concentration until each polymer binding site is occupied by a dye molecule. With further reduction in the amount of polymer, dye is released into solution and the α band begins to reappear.

Although the aggregation theory explained many facts, the failure of some polymers to elicit α or monomer bands even with considerable polymer excess, and of other polymers to elicit γ or higher aggregate bands with equivalent amounts of polymer and dye led Michaelis[5] to subsequently doubt the aggregation theory as a general explanation of metachromasy.

Recently an alternative model has been put forward by Steiner and Beers[6] and Beers, Hendley, and Steiner[7] to account for the differences in the color of AO when

bound to different amounts of polymer. These authors refer to the state of the bound dye when each binding site is occupied by a dye as Complex I and to the dye when there are many sites available per dye as Complex II. Complex I therefore exhibits β and γ bands while Complex II exhibits the α band. In this model the spectrum of Complex I differs from that of Complex II because the dye is bound to a chemically different binding site, e.g., in the case of the polynucleotide polyadenylic acid the dye might be bound to the adenine bases in Complex I and to the doubly charged terminal phosphates in Complex II. For each polymer it is necessary to assume that the number of Complex I type sites is always much greater than the number of Complex II sites so that in mixtures containing equivalent

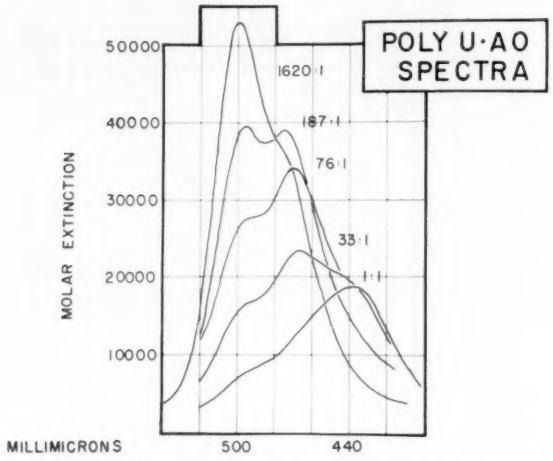

FIG. 2.—Spectra of AO bound to polyuridylic acid (Poly U). This series of spectra shows the transition from the Complex I-γ band to the Complex II-α band brought about by increasing the relative amount of polymer to which the dye is bound. As the number of polymer sites per dye is increased from 1 to 33 and 76, the γ band falls and is replaced by a β band, which in turn is replaced by the α band at still higher polymer concentrations. Heparin produces a similar array of spectra, but the number of sites per dye required to produce any given spectrum is much greater than for Poly U.

amounts of polymer and dye Complex I type spectra predominate. It is also necessary to assume that the Complex II sites bind more strongly so that with considerable polymer excess, when there are a sufficient number of Complex II sites available to bind all the dye, the Complex II type spectra will predominate. In their most recent publication[6] these authors are careful to point out that this model is to be considered only as a possible alternative to the model involving nearest neighbor interactions. They feel that it is not at present possible to decide conclusively between these two alternatives on the basis of their published data.

We have examined the spectrum of AO when mixed with seven different polymers, over a wider range of polymer to dye ratios than Michaelis, and over a wider range of ionic strengths than Beers and Steiner. Under these conditions, in contrast

to the observations of these previous workers, we have observed β and/or γ bands in mixtures containing one dye per binding site, and α bands in mixtures containing many binding sites per dye, for all seven polymers. An additional new finding is that the number of excess polymer sites required to develop the α band of AO varies widely from polymer to polymer: the band is about one-half developed in desoxyribonucleic acid (DNA) with 6 sites per dye whereas with polyphosphate 1600 sites per dye are required. In order to explain in detail all of the present findings in terms of the model of Beers and Steiner, a number of *ad hoc* hypotheses as to the types of sites available, and the relative numbers and binding strengths of each type of site, are required in each case. On the other hand, all of the findings may be interpreted quantitatively in terms of the aggregation theory with the introduction of a single new concept.

This new concept is based on the aforementioned observation that although all of the polymers exhibit α, β, and/or γ bands, the number of sites per dye required to

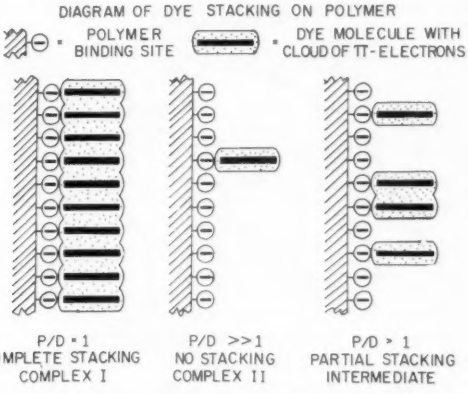

FIG. 3.—Schematic representation of the aggregation of dye molecules bound to the surface of the polyelectrolytes.

develop the α band differs from polymer to polymer. This may be seen in Figure 4 in which the extent of development of the α band of Complex II is plotted against the number of polyanion sites per dye (P/D), on a semilog scale, for the various polymers. All of the polymers thus appear to stack the dye molecules but differ in the extent to which dilution by excess binding sites tends to unstack them. These differences bear a close similarity to the variations in the effect which dilution exerts on the unstacking of AO aggregates (in solution in the absence of polymer) which may be brought about by changes in the ionic strength or temperature of the medium. Such changes in solution are reflected by changes in the association constant of the dye. By analogy we wish to introduce a numerical parameter, the dye-stacking coefficient, to express the characteristic tendency of a polymer to promote the reversible association, or stacking, of dye molecules bound to its surface.

The analogy between the stacking of dyes in solution and polymer-bound is not complete because molecules in solution are free to occupy any volume element

whereas the polymer-bound molecules are constrained to occupy positions on the binding sites. This constraint also limits the maximum concentration of bound molecules to that state in which each site is occupied by a dye molecule. As a consequence of these differences the equilibrium constant expressions developed for solution chemistry are not directly applicable to the case where molecules may occupy only a finite number of binding sites arranged in a regular array. However, since the number of sites and dyes may be counted, the problem may be treated by probability theory. An equation has been developed from statistical postulates which is analogous to an association constant expression. This stacking equation expresses the fraction of Complex II (unstacked molecules) as a function only of the concentration of dye on the surface of the polymer (dyes per site) and the dye-stacking coefficient. The solid curves in Figure 4 show the fit of this equation to the experimental data.

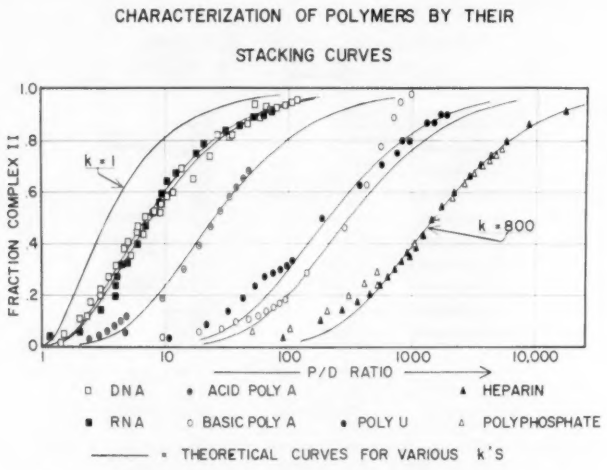

FIG. 4.—Theoretical and experimental stacking curves. Ordinate: Fraction, F, of unstacked dye molecules (Complex II). Abscissa: Number of binding sites per dye (P/D). Solid curves are plots of equation (3) for the k values listed in Table 1. Experimental values of F are calculated from spectral data using equation 2, at measured ratios of polymer to dye.

Experiments were carried out with AO (National Aniline Co.) recrystallized as the free base from methanol/KOH/water. All solutions of dye and polymers were routinely buffered at pH 6.7 with $M/1{,}000$ cacodylate buffer. Experiments at higher ionic strengths were carried out in 0.1 M and 1.0 M NaCl at pH 6.7 in the $M/1{,}000$ cacodylate buffer, and at lower pH with $M/1{,}000$ acetate-acetic acid buffer, pH 4.9. Absorption spectra were measured at room temperature (22°C) from 220 to 550 mμ with a Model 14 Cary recording spectrophotometer. Spectra of the free dye and of dye plus polymer were obtained by serial addition of increments of polymer solution to a fixed amount of dye in a stoppered cuvette. Polyadenylic acid (Poly A) and polyuridylic acid (Poly U) were synthesized in this laboratory using polynucleotide phosphorylase isolated from *Azotobacter vine-*

landii.[8] Ribonucleic acid (RNA) from mouse ascites tumor cells was a gift of Dr. John S. Colter. Salmon sperm desoxyribonucleic acid (DNA) (California Foundation), heparin (Lipo-Hepin, Darwin Laboratories), and sodium polyphosphate glass (Poly P) (Monsanto) were obtained commercially. Dye and polymer solutions were prepared from weighed amounts except for heparin, and the concentrations of the polynucleotide solutions were checked by comparing extinction coefficients with known values.

The spectrum of free AO in dilute solution (2×10^{-5} *M*) is shown in Figure 1. When polymer is added to this solution the spectrum changes in a continuous fashion as dye molecules are adsorbed to the polymer, until one polymer site has been added for each dye molecule. Beyond this point further additions of polymer cause spectral changes which are much more gradual and which are in the opposite direction. There appears therefore a sharp break in the mixing curves at the 1:1 point which serves to define Complex I and its spectrum quantitatively. The ratios of polymer charged groups per dye molecule at which this break appears are shown in Table 1 as calculated by independent measurements of polymer and dye

TABLE 1

COMPARATIVE DATA ON THE STACKING TENDENCY OF AO WHEN BOUND TO DIFFERENT POLYMERS

Polymer	P/D ($\alpha=\beta$)	k	α_{max}	β_{max}	γ_{max}	E_I	E_{II}	P/D (max α sup.)
DNA	5	2.9	502	466	...	15000	51000	0.97
RNA	7	3.3	506	465	...	12000	58000	1.05
Poly A (acid)	17	12.3	502	468	...	16500	45500	...
Poly U	190	109	498	472	440	5000	55000	1.05
Poly A (basic)	285	161	502	464	...	12000	58500	0.93
Heparin	700	787	502	470	450	9500	53000	1.28
Poly P	1580	826	492	...	455	12500	55500	2.18

P/D ($\alpha = \beta$) is the P/D at which the α and β bands are of equal height. k is the stacking coefficient. α, β, and γ max are the wave length maxima in mμ of the α, β, and γ bands. E_I and E_{II} are the molar extinctions of complex I and II, respectively, at 492 mμ for poly *P* and 504 mμ for all other polymers. P/D (max α sup) is the P/D at which the α band is maximally suppressed, as calculated from the molar extinction coefficients of the polynucleotides, and the weights of heparin and poly *P* used.

concentrations. The 1:1 stoichiometry is particularly good in the cases of the polynucleotides where the polymer concentrations were accurately known. At higher ionic strengths the sharp break at 1:1 was not obtained and the spectra behaved as if the binding of dye to polymer were incomplete. The dissociation of dye-polymer complexes at high ionic strengths has been described previously.[3] As this effect tended to obscure the stacking effects, the work referred to below was carried out at $\mu = 1/1{,}000$.

The spectra of Complex I fell into two classes: DNA, RNA, Poly A (basic), and Poly A (acidic) exhibited β band spectra while heparin and Poly U showed γ band spectra. Poly P was intermediate. These spectral types are shown in Figures 1 and 2 and the λ_{max} are shown in Table 1. Presumably those polymers which have β band spectra permit the formation only of dye dimers, while those with γ band spectra permit formation of taller stacks.

When polymer is added beyond the 1:1 point the spectra change continuously toward the α band type characteristic of Complex II. The intermediate spectra fall into two types: DNA, RNA, Poly A (acidic) and Poly A (basic) develop bimodal spectra consisting of α and β bands. Poly U and heparin develop trimodal curves corresponding to the simultaneous presence of α, β, and γ bands (Figs. 1, 2). The appearance of a β band as an intermediate stage in the transition from the γ band to the α band in Poly U and heparin provides strong evidence in favor

of the aggregation theory. If γ bands represent tall stacks and α bands, monomers, then it would be expected that the β band representing dimers should appear as an intermediate in the process of the dissociation of the larger aggregates into monomers.

Since the α band corresponds to monomers and the β band to dimers, the state in which the α band and β band are of equal height approximates the condition in which half of the dye molecules are dissociated into monomers. The number of binding sites per dye required to obtain this condition is thus a measure of the tendency of the polymer to promote dye stacking. The polymers examined have been ranked according to this measure in Table I. A further strong argument in favor of the aggregation theory is that this measure of stacking tendency is correlated with the spectra of Complex I. Those polymers which exhibit γ bands, indicating a tendency to favor tall stacks, in general show higher stacking tendencies as measured by this completely independent method.

The λ_{max} and extinction coefficients of the α bands are given in Table 1. In all cases except Poly P there is a small bathochromic shift of about 10 mμ from the α band of AO in solution. Similar bathochromic shifts have been described by Michaelis[8] for toluidine blue, and by DeBruyn and co-workers[9] for a large number of cationic dyes, both metachromatic and normal. These workers attribute this shift simply to the binding of dye to the polymer site and not to dye-dye interaction on the polymer surface.

In developing a quantitative theory to account for the shift from the β and γ band spectra of Complex I to the α band spectra of Complex II we shall start from the assumption that a dye molecule bound to a linear polymer site aggregates with, or stacks onto, only those dye molecules on adjacent polymer sites. If both adjacent sites are empty the molecule cannot stack and is therefore a "monomer" exhibiting the α band spectra of Complex II. When every site is occupied by a dye molecule all dyes are stacked and the spectrum consists of pure β and γ bands of Complex I. As the amount of polymer is increased empty sites become available. Since the dye molecules will tend to distribute themselves evenly among all of the available sites, some of them will occupy sites without neighbors, so that a mixture of Complex I and II results. As the number of empty sites is made very large virtually all of the molecules occupy sites without neighbors and a pure Complex II spectrum is observed. These changes are shown diagrammatically in Figure 3.

Suppose the distribution of dye molecules among available sites were completely random. Then, from probability considerations, it can be shown that the fraction, F, of unstacked dye molecules (Complex II) would be related to the ratio, P/D, of the number of polymer sites to the number of dye molecules by equation (1).

$$P/D = (1 - F^{1/2})^{-1} \tag{1}$$

In Figure 4, F is plotted against P/D for equation (1) (curve labeled $K = 1$) and for the experimental data. The experimental values of F were computed from the molar extinction coefficients (at the Complex II maximum) of pure Complex II, (E_{II}), pure Complex I, (E_I), and the solution having a given P/D, (E) by equation (2).

$$F = \frac{E - E_I}{E_{II} - E_I} \tag{2}$$

Equation (2) is equivalent to that used by Zanker in calculating the degree of association of AO in solution, and assumes the presence of only the two species Complex I and II.

It is striking that all of the experimental curves are similar in shape to the curve of equation (1) but are displaced to higher P/D's: the fraction of stacked molecules is always greater than predicted by equation (1). This demonstrates on an absolute numerical basis that dye molecules are not randomly distributed among the available sites but prefer to occupy sites adjacent to one another, where they are stacked. This preference may be treated quantitatively by supposing that the probability, $P(1)$, that a dye molecule will occupy a particular site with a nearest neighbor, is greater than the probability $P(2)$, that it will occupy a particular site without a nearest neighbor. The ratio $P(1)/P(2)$ is thus a measure of the inherent tendency of the dyes to stack which is independent of the relative numbers of sites and dyes. This ratio, $P(1)/P(2)$, is the quantity which will be referred to as the stacking coefficient, and by assuming that it is a constant, k, an equation may be developed from probability considerations relating k to P/D and F:

$$P/D = (1 - F^{1/2})^{-1} + (k - 1)F^{1/2}(1 + F - F^{1/2})(1 - F^{1/2})^{-1} \qquad (3)$$

Although the derivation and properties of equation (3) will be treated in detail in a separate communication, it can be readily shown to satisfy a number of intuitively obvious conditions. When each polymer site is filled ($P/D = 1$) all molecules are stacked ($F = 0$). As the number of sites becomes very large ($P/D \rightarrow \infty$) all molecules become unstacked ($F \rightarrow 1$). When there is no preference for adjacent sites ($k = 1$) equation (3) reduces to the random distribution equation (1). When there is preference for adjacent sites ($k > 1$) the amount of dilution by empty sites (P/D) required to achieve a given degree of unstacking (F) is greater than for the random distribution. It might also be mentioned that equation (3) was developed for a polymer of infinite chain length. End effects in polymers of finite length will result in an increased number of unstacked molecules, so that experimental F values for finite polymers will lie above the stacking curves of equation (3).

In Figure 4 the solid lines are curves of equation (3) fitted to the experimental data at $F = 0.5$, and in Table 1 are listed the k values of the polymers corresponding to these curves. It should be noted that the polymers fall into the same order whether ranked according to k or the α and β band method previously described. The good fit of the experimental data to the equation, with the possible exception of Poly A (basic), and the wide range in magnitude of k, suggest that k may prove to be a useful new parameter for the characterization of polyelectrolytes.

A variety of factors may determine the effect which a particular polymer has upon the stacking tendency of dyes bound to its surface. Among these factors could be the extent to which the cationic charge of the dye is neutralized, the change in the dielectric constant in the vicinity of the dye, and the extent to which thermal agitation of the dye is reduced because of binding. Perhaps the most important factor, however, would be the relative orientation of dyes bound to adjacent sites, and the rigidity with which this orientation is maintained. It may be speculated that polymers whose binding sites are free to assume optimal positions for the stacking of dye bound to them will have high stacking coefficients, while those whose binding sites are rigidly held in positions less than optimal will have lower stacking

coefficients. DNA, which is presumed to have a rigid two-stranded configuration in solution, has the lowest stacking coefficient observed. Heparin and polyphosphate, which are presumably flexible single stranded coils in solution have the highest stacking coefficients.

There is considerable evidence that Poly A undergoes a change in structure from a single-stranded coil above pH 7 to a rigid, two-stranded helix below pH 5. The stacking coefficient is high at pH 7 but low at pH 5 for Poly A. This gives further support to the idea that a high stacking coefficient corresponds to a flexible arrangement of binding sites, while a low one corresponds to a rigid arrangement, as found in a multistranded helix.

The structure of RNA is the subject of some controversy. Our sample has a stacking coefficient close to that of our sample of DNA, and we may infer that the two polymers have the same degree of molecular rigidity. Work is in progress to determine whether RNA and DNA samples from various biological sources, prepared by different methods, have similar stacking coefficients. Work is also in progress with metachromatic dyes other than AO. It has already been ascertained that the stacking coefficient of RNA, when measured with the dyes acridine yellow and coriphosphine O, is identical with that determined by means of the binding of AO.

The authors wish to acknowledge the able assistance of Mr. John Chen in carrying out many of the experiments.

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SEARCH FOR UNSTABLE CO_2 FIXATION PRODUCTS IN ALGAE USING LOW TEMPERATURE LIQUID SCINTILLATORS

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Introduction.—In early studies of photosynthesis in algae[1] a variety of killing procedures was examined and compared. Since the various methods studied did not give very different results, killing with boiling ethanol followed by extraction with water was adopted as a standard procedure. In the work published in 1957–

58[2, 3, 4] a higher total fixation of C^{14} seemed demonstrable when photosynthesizing green algae (*Scenedesmus* or *Chlorella*) were killed by pouring their suspension into deeply cooled (−20° to −40°C) organic solvents (acetone or ethanol) than when they were killed by the usual procedure employing boiling ethanol. The percentage difference between the two killing methods (hot vs. cold) was greater in shorter periods of photosynthesis.

Preliminary killing experiments performed at temperatures between 0° and −70° showed that the most reproducible results were obtained between −20° and −40°. At temperatures near 0° one does not observe great differences from the normal procedure, and at the lower temperatures (−70°C) ice formation makes the manipulation difficult. A great part of the difference in the activity fixed in the cold and warm killing experiments could be caught as evolved CO_2 from the cold extract during manipulation. Because of what appeared to be suitable control experiments, this was attributed to the decomposition of an unstable compound different from bicarbonate, which could not survive plating or chromatography.

An attempt was made to learn more of this unstably bound CO_2 using the combustion method of Wilzbach and Sykes.[5]

The *Scenedesmus* suspensions were poured into cold acetone or ethanol (−30°) and after addition of 1 ml of acetic acid for each 10 ml of original suspension to remove the excess of bicarbonate, an aliquot was taken by precooled micropipettes and injected into a very cold (−183°liquid oxygen) bomb with a copper-copper oxide mixture. After warming the tube to −45°, evacuating for a fixed short period of time, and sealing, the organic material was burned and the resulting radioactive carbon dioxide measured with a vibrating reed electrometer. By this method the "total fixation" was estimated. An equal aliquot spread on an aluminum plate and burned in a second bomb gave what was called the "stable fixation." The difference between the two measurements was attributed to "the unstable compound." The results were interpreted as confirmation of some of the differences described in the earlier work. No fixation was obtained in the experiments with the algae killed in acetone before the radioactive bicarbonate was added nor with the algae exposed to radioactive bicarbonate in the dark and flushed for 70 min with nitrogen. These results did not reveal the nature of the unstable compound or compounds, and in fact, left doubt as to their very existence.

For a more extensive examination of the unstably bound CO_2, the determination of the radioactivity in liquid scintillators at −15°C seemed to offer an advantage over the very time-consuming combustion method of Wilzbach and Sykes. This method had been proved[6] to give dependable results with the suspensions of inorganic compounds, homogenized tissues and bacteria.

Experimental.—Scintillation counting had never been used for the counting of algae suspensions, therefore, the following factors were studied before the method was applied to the low temperature killing experiments.

(*a*) the relative efficiency of this scintillation counting compared to that of Geiger-Mueller counting,

(*b*) the time course of the response of the scintillator containing a radioactivity sample (including bicarbonate),

(*c*) counting efficiency as a function of the algae content of the scintillator liquid,

(*d*) the effect of the temperature on counting.

For the determination, 20 ml bottles were used containing as a scintillator 10 ml of 2,5-diphenyloxazole (PPO) solution in toluene (5 g in 1 kg). Three ml of absolute ethanol was added to each bottle to facilitate the solution of the aqueous suspension of algae. The liquid scintillation counter used in this study was a coincidence anticoincidence counter described earlier.[7] The determinations were made at $-15°C$. For the estimation of the relative counting efficiency of the scintillation counter with respect to the Geiger-Mueller counter, solutions of radioactive benzoic acid, sodium bicarbonate, and sucrose were used.

The results show that the counting in liquid scintillators offers a quick and suitable method for the estimation of the activity of the solutions of radioactive bicarbonate, benzoic acid, and sucrose in the presence of algal suspensions. The results are reproducible for several dilutions. The counting efficiency is 80 per cent for benzoic acid and sucrose. The activity of samples kept at $-15°C$ does not change over a period of several hours. The apparent decrease in count caused by the presence of the algae suspension used in later experiments does not exceed 5 per cent.

This method was applied to the investigation of the possibility that the radioactivity found in previous experiments and ascribed to the presence of unstably bound CO_2 might be caused by a residue of the radioactive bicarbonate added to the suspension of photosynthesizing algae. To explore this possibility three kinds of experiments were done in which bicarbonate was added to:

(1) normal photosynthesizing algae,

(2) the algae kept in the dark,

(3) the suspension of killed algae.

For these experiments three different post-killing conditions were applied for each of the two organic solvents;

(*a*) the suspension of algae was kept at $-45°C$,

(*b*) the suspension of algae was kept at $-45°C$ and nitrogen was bubbled through it for 15 min,

(*c*) the suspension of algae was kept at room temperature.

The conditions of the experiments were nearly the same as in the previous[2, 3, 4] experiments except that the quantities used were only $^1/_{10}$ as large. One ml of 2 per cent or 4 per cent suspension of *Scenedesmus* was preilluminated for 10 min in the presence of 1 per cent CO_2, then swept with N_2 for 3 min. Afterwards 4 or 8 μc of $NaHC^{14}O_3$ was added and the mixture was allowed to stand for 30 seconds. Then the algae were killed with acetic acid acidified solvents—4 ml of ethanol (for the 2 per cent suspensions) to give 80 per cent ethanol, or 9 ml. of acetone (for the 4 per cent suspensions). A 4 per cent suspension of algae was used in the experiments with the acetone killing in order to produce a 0.4 per cent suspension of algae (as in the alcohol killing) in 90 per cent acetone solution. (The latter concentration was required because 80 per cent acetone freezes at $-45°C$.)

In the cold killing experiments, (*a*) and (*b*), the algae were killed with the solvents cooled in liquid nitrogen to $-60°C$ and placed immediately in an acetone-dry ice bath at $-45°C$. In experiments (*c*) the killing was done with the solvents at room temperature. In all cases the 100 λ samples were taken as soon as possible; in (*a*) and (*b*) with pipettes precooled to $-45°C$. In experiments (*b*) after the

taking of the first sample, nitrogen was bubbled through the suspension for 15 min.

Samples were taken every 15 min during the first 2 hours, the next one at 3 hours and another one the next day. The results of these experiments are represented in Figures 1–6. One can see that at room temperature bicarbonate has disappeared

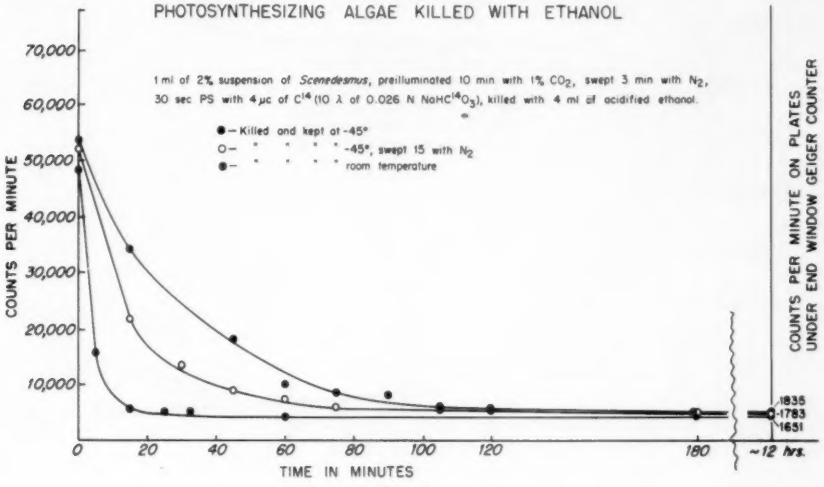

FIG. 1.

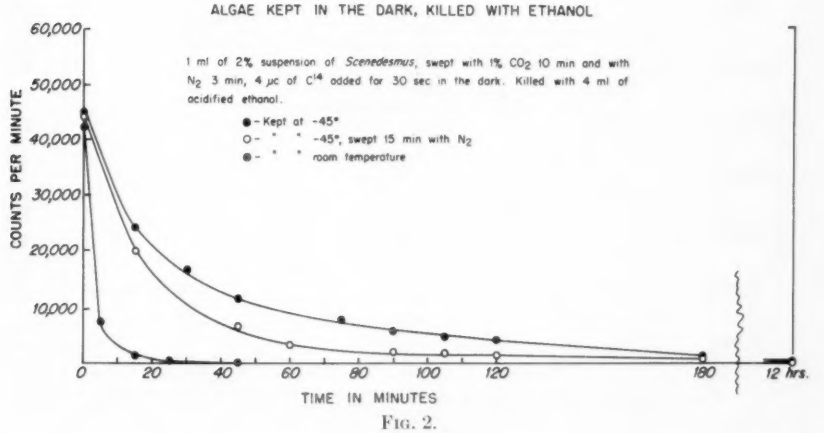

FIG. 2.

nearly completely after $^1/_2$ hour from the ethanolic suspensions and somewhat more slowly from the acetone suspensions. The bubbling of N_2 for 15 min does not completely remove bicarbonate, although it diminishes its quantity. In ethanol solutions the differences between the radioactivity of the suspension of algae kept at $-45°C$ flushed with nitrogen and that not flushed are much smaller than that

in acetone solution. This is probably caused by the greater viscosity of alcoholic solutions at $-45°C$, from which CO_2 is not so easily expelled as from the acetone solutions which are completely liquid at that temperature. In the experiments in which the bicarbonate was added to the suspension of dead algae, one observes a

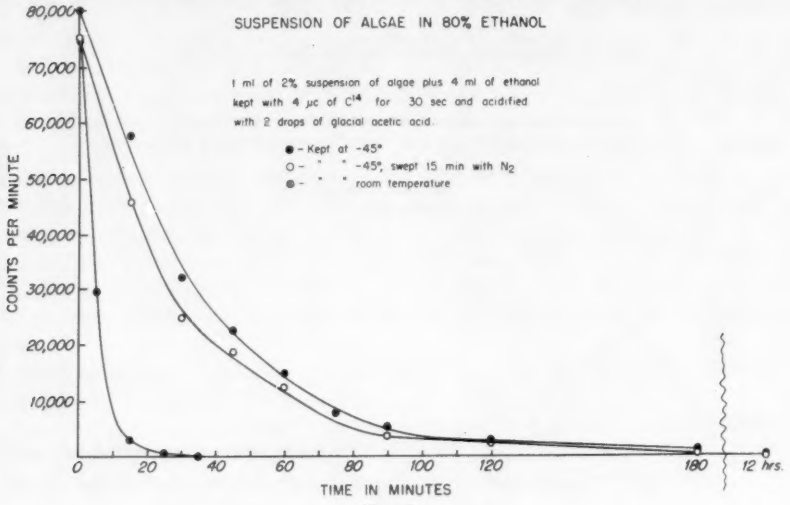

FIG. 3.

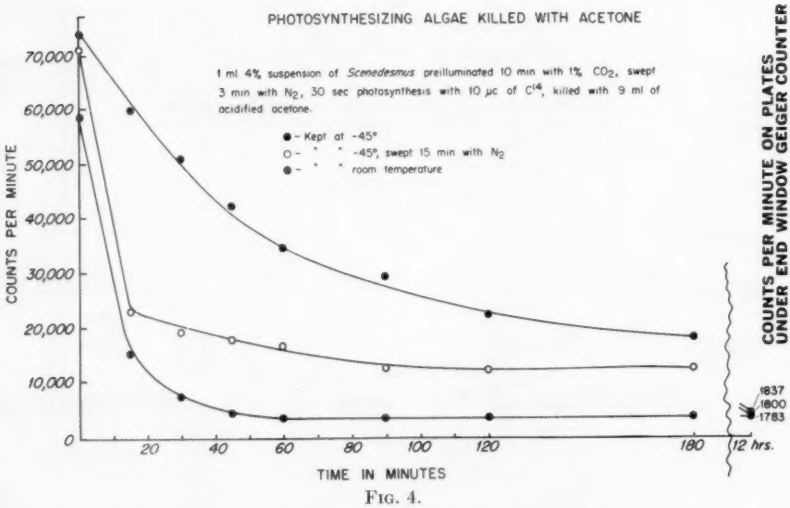

FIG. 4.

complete disappearance of the radioactivity in ~12 hours. In the dark experiments in which the suspension of algae was flushed for 10 min with 1 per cent CO_2 and for 3 min in the dark with N_2, and the radioactive bicarbonate added for 30 sec before killing, one can observe a very small fixation.

Results.—Figures 1, 2, and 3 show that in the algae killed with alcohol and kept at −45°C practically all the bicarbonate disappears in 3 hours. This is not the case of acetone solution in which the radioactivity takes not less than 6 hours at −45°C to reach its final value (Fig. 4, 5, and 6).

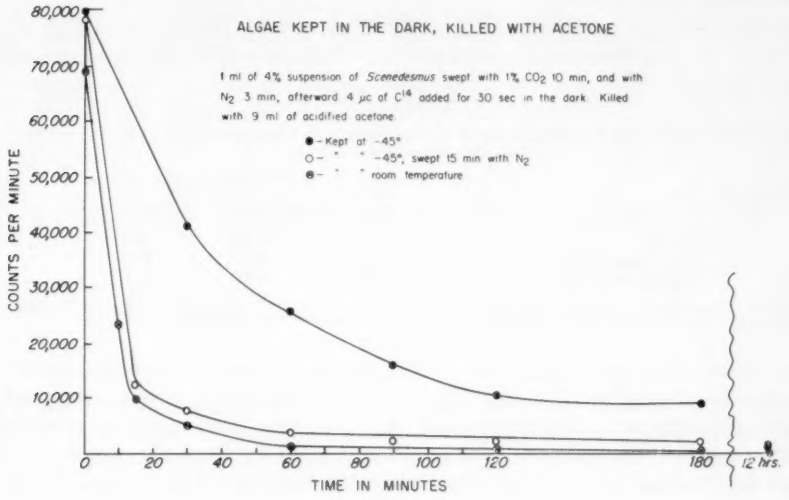

FIG. 5.

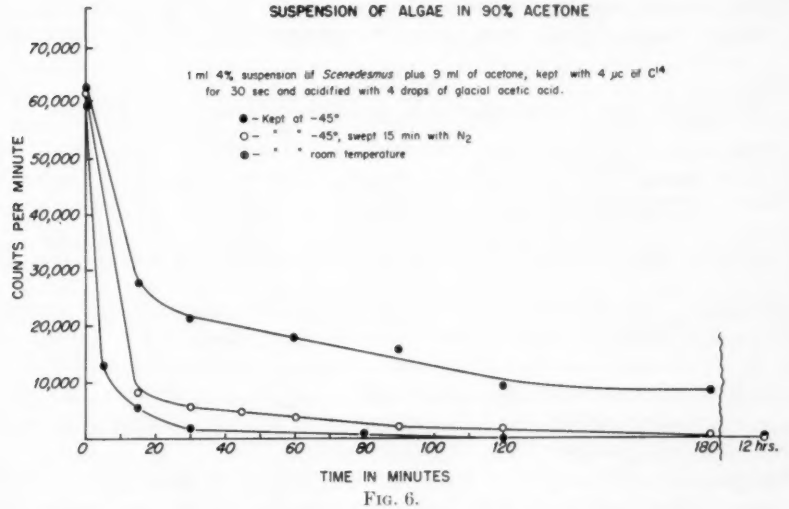

FIG. 6.

Separate experiments not including algae showed that bicarbonate is considerably more stable in acetone than it is in alcohol at −45°C or lower. Beyond this the acetone volumes were larger than the alcohol volumes and the rate in bicarbonate escape was correspondingly slower.

Contrary to the previous results,[2, 3, 4] it appears that in normal photosynthesis the stable fixation is the same no matter at what temperature the algae are killed. The results in Tables 1 and 2 show that in all cases the stable fixation measured in the scintillation and in the Geiger-Mueller counters was the same within the limits of accuracy of the methods.

These results fail to confirm the existence of an unstable product of early photosynthetic fixation persisting for some time at −30°C or −45°C different from and

TABLE 1

EFFECT OF SOLVENT AND TEMPERATURE ON TOTAL APPARENT PHOTOSYNTHETIC CO_2 FIXATION BY TWO COUNTING PROCEDURES

Solvent →	No. of Counts per Aliquot		
	Acetone	Ethanol	
	In Scintillation Counter at −15		In G. M. on Plates
Temperature			
Boiling	7936	8192	2750
Room temperature	7936	8448	2650
Low temperature, −35°	7680	8960	2430

1 ml of 1% suspension of Scenedesmus preilluminated 10 min. with 1% CO_2 and swept 3 min. with N_2; photosynthesis for 30 sec. with 4 μc of C^{14}; killed with 5 ml. of organic solvent; 100 λ counted.

more stable than bicarbonate. Our experiments also failed to confirm the differences previously found in the stable fixation of radioactive bicarbonate which appeared to depend on the temperature of the killing of photosynthesizing algae. On the contrary they proved that the stable fixation for the same suspension of algae for the same period of time, and the same quantity of added bicarbonate, is independent of the solvent used and of the temperature of the killing.

All the previous experiments can now be accounted for in terms of differences in the rate of escape of $C^{14}O_2$ from the various preparations. There remains the

TABLE 2

EFFECT OF SOLVENT AND TEMPERATURE ON TOTAL PHOTOSYNTHETIC $C^{14}O_2$ FIXATION

Solvent →	No. of Counts per Aliquot		
	Acetone	Methanol	Ethanol
Temperature			
Boiling	9430	10320	10167
Room temperature	9070	10360	8010
Low temperature, −60°C	9370	9190	10160

2 ml of 2% suspension of *Chlorella* preilluminated for 10 min with 1% CO_2 and swept for 3 min with N_2, 15 sec photosynthesis with 20 μc of C^{14}, killed with 8 ml organic solvents 100·λ counted in Geiger-Mueller counter.

experiment in which the quantity of the apparent "unstably bound" CO_2 is dependent upon the immediate prior illumination of the algae (Expts. 2 & 7, Table 1, of Ref. 3). This seems now best interpreted in terms of a production of alkali as a result of the photosynthesis immediately preceding the administration of $C^{14}O_2$. Again independent experiments in the absence of algae, with added quantities of alkali, produced the expected slowing of the escape of $C^{14}O_2$.

The possibility still remains that an "active" CO_2 may be present but in small amounts. This would be required if it is more or less stable than bicarbonate; if its stability is similar to that of bicarbonate these experiments could not distinguish it even in large amounts.

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RADIOACTIVE FALLOUT PARTICULARLY FROM THE RUSSIAN OCTOBER SERIES

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Communicated May 29, 1959

Introduction.—Radioactive fallout has been extensively studied and reported upon[23, 25, 26, 30] and, in general, although certain questions remain unanswered, the broad characteristics of its behavior have been established.

The stratosphere, the top fifth of the atmosphere lying above about 40,000 feet, plays an extremely important role. In fact, the fallout from megaton yield weapons occurs very largely from it while the troposphere is the medium which disseminates the fallout from kiloton detonations; thus, speaking broadly, stratospheric debris is from H-bomb detonations and tropospheric fallout is from A-bombs. It is not that the yield of the detonation is determinative, but rather that the altitude to which the fireball rises determines the fallout rates. The megaton yield fireballs are so enormous that they stabilize at levels only above the tropopause—the imaginary boundary layer dividing the upper part of the atmosphere, the stratosphere, from the lower part, the troposphere—while the kiloton yield fireballs stabilize below the tropopause. The tropopause normally occurs at something like 35,000 to 55,000 feet altitude, although it depends on season and location. In other words, low yield bombs fired in the stratosphere would be expected to give the same fallout rates as high yield weapons do when fired in the troposphere—or on the surface. There is some small part of the fallout for megaton yield explosions which does not reach the stratosphere.

The stratospheric debris descends very slowly unless, of course, it is attached to particles so large as to fall in the first few hours, as in the case of surface bursts. This paper is concerned only with the worldwide fallout—that is, the fallout which does not occur in the first few hours—and excludes the local fallout which constitutes the famous elliptical pattern which is so hazardous because of the intensity of its radiation at early times but which, in test operations, is carefully restricted to test areas. It should be noted that the local fallout is a principal hazard in the case of nuclear war. Most serious attention should be paid to it in civilian defense programs.

The worldwide fallout from the stratosphere occurs at a slow rate. The rate of descent of the tiny particles produced by the detonations is so small that something like five years appears to be the average time those from equatorial shots spend before descending to the ground, corresponding to an average annual rate of about twenty per cent of the amount in the stratosphere at any given time.

A principal point of this paper is to present evidence for the theory of Dr. E. A. Martell[1] that shots conducted in polar latitudes have a much shorter stratospheric residence—of about one year. This earlier precipitation of Russian debris has reduced the estimated residence time for equatorial fallout to five years because the method is to compare the observed fallout rate with the stratospheric inventory which necessarily becomes smaller by the earlier subtraction of the Russian polar debris.

It is not clear as to just how the tiny stratospheric particles do finally descend. It seems possible that the general mixing of the stratospheric air with the tropospheric air, which occurs as the tropopause shifts up and down with the season as well as what is brought about by the jet streams, constitute the main mechanisms. The descent of the stratospheric fallout apparently is never due to gravity but rather to the bulk mixing of stratospheric air with tropospheric air which brings the radioactive fallout particles down from the stratosphere into the troposphere where the weather finally takes over. This mechanism makes the percentage rate the same for all particles too small to fall of their own weight—and the same as would be expected for gases, providing some means of rapidly removing the gases from the troposphere exists, so the reverse process of troposphere to stratosphere transfer would not confuse the issue.

The worldwide fallout from the stratosphere descends very slowly, corresponding to residence times of 1 to 5 years depending on latitude, whereas the troposphere has a mean residence time of only one month with the air in the lower 10,000 feet being washed clean on the average about every three days. Between 10,000 feet and the tropopause, which is at something like 35,000 north of 30°N and at about 55,000 feet in equatorial regions between 30°S and 30°N, the residence time is perhaps 45 days for a half time for the troposphere as a whole of about one month. This short time means that radioactive fallout which is injected into the troposphere is restricted to the general latitude of the detonations for the reason that the residence time is so short that it doesn't have time to mix appreciably latitudinally. Similarly, the 1 year residence time for polar stratospheric debris means that it falls out mainly in the Northern Hemisphere because the inter-hemispheric mixing time appears to be longer.

The principal mechanism for precipitation of fallout is rain. Apparently, the tiny fallout particles hit cloud droplets and stick to them, because the particles are so small (perhaps a few hundred atomic diameters) and thus are subject to the violent random jiggling due to collisions with air molecules, the Brownian motion. In fact, for a particle one micron in diameter, Greenfield[2] calculates that the mean residence time in a typical cloud of water droplets of 20 microns diameter would be between 50 and 300 hours, that for a particle of 0.04 micron diameter it would be between 30 and 60 hours, and that for a particle of 0.01 micron diameter it would be between 15 and 20 hours. The theory calculates the diffusion due to the Brownian motion and says that it is in this way that the fallout particles and the

cloud droplets occur. Of course, once the fallout particle is taken into a droplet it is likely to be carried along with the normal weather processes from which rains result.

There is essentially no worldwide fallout in the absence of rainfall; i.e., in desert regions—except for a little that sticks to tree leaves, blades of grass, and surfaces in general, by the same type of mechanism Greenfield describes in the case of clouds. Thus it is that the moisture in the troposphere assures the short lifetime of the tropospheric worldwide fallout particles and, that stratospheric air which contains essentially no moisture* has a much longer residence time. When stratospheric air finally does descend into the troposphere, the tropospheric moisture proceeds to clean it up by the Greenfield mechanism of accretion on to cloud droplets. Actual samples of cloud droplets taken at Mt. Washington, New Hampshire,[3] have shown strontium-90 content per unit volume of water so derived in good agreement with those observed for rain in the same general area. Also, Suess[4] reports that at La Jolla (California) where there is little rain, an open tub, collecting sea spray at the end of a pier, showed strontium-90 content in general keeping with the theory. Of course, the same jiggly diffusive motion which brings the tiny fallout particles into contact with the droplets in a cloud will make contact with leaves of grass and trees, etc., possible as one does observe as previously mentioned.

Russian October Fallout.—The intensive series of bomb tests fired by the USSR during last October (1958) affords a unique opportunity to test whether stratospheric radioactive fallout from injections made at polar latitudes differs appreciably in distribution or fallout rates from that due to equatorial explosions, such as the United States and United Kingdom have fired. The Russian October series is estimated, on the basis of assumptions previously described,[5] to have added about 12.5 to 15 megatons† equivalent of fission products to the stratosphere, whereas the previous inventory on the same basis was about 18 megatons equivalent, if we anticipate the result, demonstrated later, that polar debris falls out in about 1 year instead of 5. The Russian addition amounted to a sudden increase of about 150 per cent in the Northern Hemisphere, if we take the previous stratospheric burden to have been uniformly distributed. In addition to the suggestion of Martell[1] that nuclear explosions conducted in the polar latitudes and which inject radioactive fallout into the stratosphere may have a stratospheric residence time of about one year, a time much shorter than for those tests conducted in equatorial latitudes,[5] the Department of Defense[6] has tentatively concluded on the basis of data which it has collected, together with the Atomic Energy Commission's stratospheric balloon data, that the residence time for Polar shots is about one year or less and that equatorial debris has a residence time of about three years.

L. Machta[7] has emphasized that the well-established nonuniformity of the total fallout in the Northern Hemisphere with a peak in the middle latitudes might be due to a natural characteristic of the circulation of the stratosphere which concentrates stratospheric fallout in these latitudes. The author[5] has pointed out the fact that major test sites in the United States and the USSR lie in these latitudes and that tropospheric fallout from these sites which, because it is airborne only one month or so on the average and, consequently has relatively little chance to spread in the North-South direction, could contribute a band of early fallout around the earth in the general latitude of the test site which might account for a large part of the peak. Comparison of foreign with domestic soil strontium-90 data shown in

Table 1 demonstrates that the United States has about 15 mc/mi² more strontium-90 than foreign countries in the same latitudes. This shows that a part of the United States fallout was tropospheric and specifically due to tests in Nevada. The

TABLE 1

U.S. AND FOREIGN SOIL Sr^{90} CONTENTS[3, 8, 9]

	October, 1956 mc/mi²	October, 1958 mc/mi²
United States Average	17.3	47
Foreign 20–50°N Average	6.7	30–33*
Difference (U.S.—Foreign)	10.6	14 to 17

* Foreign samples for 1958 were collected in spring and summer so correlation to October was made by rain data.

data continue to raise the old question as to whether this excess of the Northern Hemisphere over the Southern Hemisphere could all be due to tropospheric debris or might be due in one way or another to stratospheric fallout.

Study of the soil data, together with recent rain data, indicates that the true situation probably lies in a combination of Dr. Martell's theory, Dr. Machta's theory, and a part of the author's model.

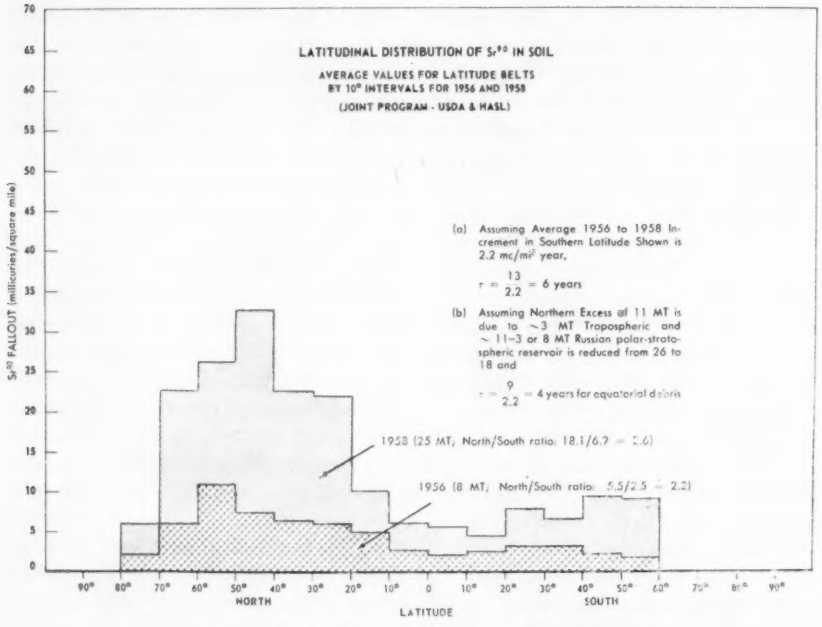
LATITUDINAL DISTRIBUTION OF Sr⁹⁰ IN SOIL
AVERAGE VALUES FOR LATITUDE BELTS BY 10° INTERVALS FOR 1956 AND 1958
(JOINT PROGRAM - USDA & HASL)
Sr⁹⁰ FALLOUT (millicuries/square mile)
(a) Assuming Average 1956 to 1958 Increment in Southern Latitude Shown is 2.2 mc/mi² year,
τ = 13/2.2 = 6 years
(b) Assuming Northern Excess of 11 MT is due to ~3 MT Tropospheric and ~11-3 or 8 MT Russian polar-stratospheric reservoir is reduced from 26 to 18 and
τ = 9/2.2 = 4 years for equatorial debris
1958 (25 MT; North/South ratio: 18.1/6.7 = 2.6)
1956 (8 MT; North/South ratio: 5.5/2.5 = 2.2)
NORTH
SOUTH
LATITUDE

FIG. 1.—Latitudinal distribution of strontium-90 in soil.

Figure 1 presents both the 1956 and 1958 soil data collected from foreign countries by Dr. Lyle T. Alexander[8–9] in the spring and early summer of both years as analyzed by HCl extraction. The 1958 data show that there was a total deposit of about 25 megatons fission energy equivalent of strontium-90 of which about 18 megatons were in the Northern Hemisphere and 7 megatons in the Southern

Hemisphere and that in the Southern Hemisphere where presumably only stratospheric fallout occurred, the rate was 2.2 megatons per year which gives an apparent residence time of about 5 years when compared with the calculated stratospheric inventory of 18 to 22 megatons in that period.

Assuming that the stratospheric fallout in the two hemispheres would be symmetrical for material injected near the equator, that is for the strontium-90 produced by the United States and the United Kingdom megaton test explosions (as indicated

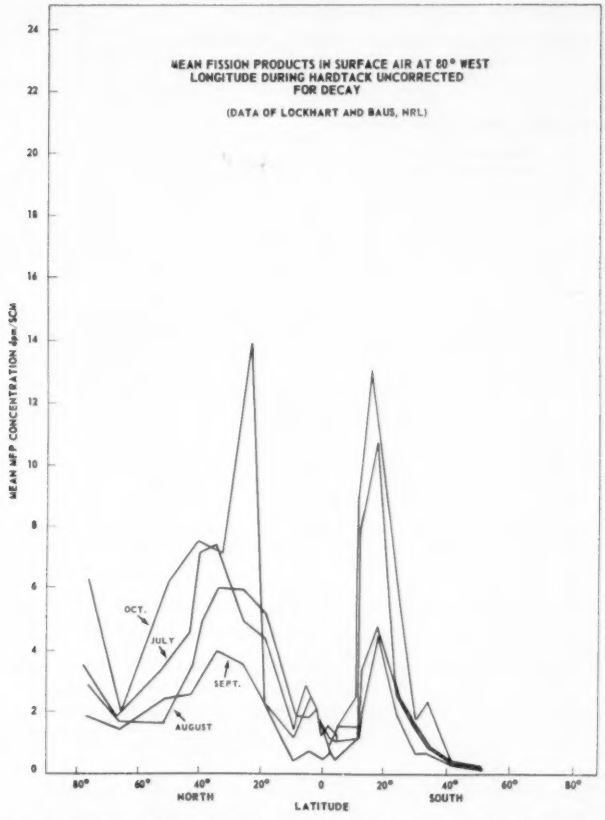

FIG. 2.—Mean fission product content of surface air during Operation Hardtack in 1958.

later by direct observation during Operation Hardtack, cf. Fig. 2) an excess of 11 megatons remain in the Northern Hemisphere. The question is whether this could be due to tropospheric fallout from test sites upwind or whether this must be due in part at least either to preferential leakage from the stratosphere in this latitude (Dr. Machta) or to Russian polar debris which, according to Dr. Martell, would preferentially settle in the Northern Latitudes because it would come down in a time so short, one year or less, that it would not have had time to spread into the Southern Hemisphere.

In the absence of firm direct measurements, an estimate of the stratospheric content is made by adding the amount of radioactivity which is injected, subtracting for the fallout and radioactive decay and calculating by difference. In this way numbers are derived which can be used to compare with the information that is available on stratospheric content and to calculate residence times from the observed fallout rates. Certain empirical rules have been used to estimate the injection numbers. These are:

A. Division between local and worldwide fallout:
   (1) Air shots—no local fallout—100% worldwide

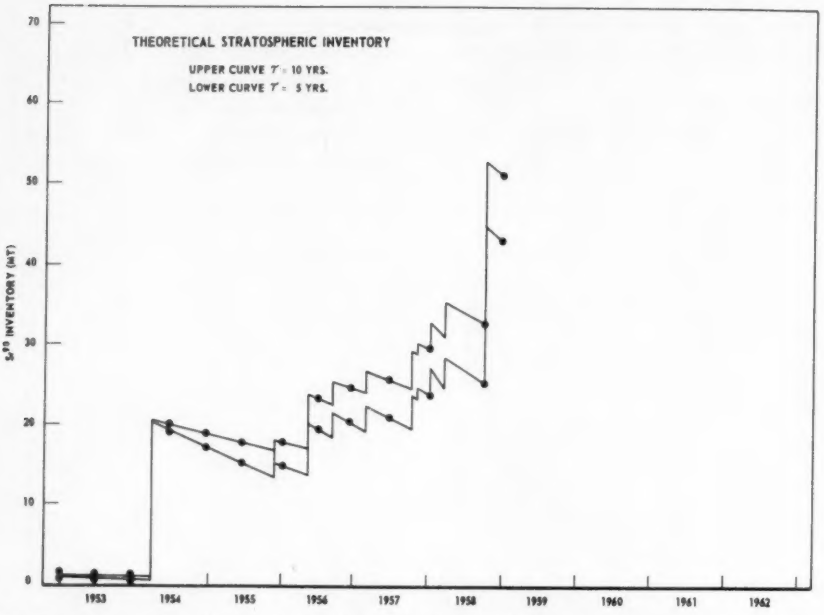

FIG. 3.—Theoretical estimates of strontium-90 in the stratosphere. Two curves are given, one for 10-year half residence time, the other for 5-year half residence time.

   (2) Surface shots on ground—80% local fallout—20% worldwide
   (3) Surface shots on water—20% local fallout—80% worldwide

B. Division of worldwide fallout between stratosphere and troposphere:
   (1) Megaton yields—99% in stratosphere—1% in troposphere
   (2) Kiloton yields—100% in troposphere

Figure 3 gives the stratospheric inventory for strontium-90 as deduced in this manner up to January 1, 1959, calculated on the basis of two assumed residence times—5 and 10 years and neglecting the Martell theory of shorter residence time for Polar shots.

The totality of nuclear releases to date, together with some over-all mention of the firing conditions is given in Tables 2, 3, and 4.

TABLE 2

UNITED STATES AND UNITED KINGDOM NUCLEAR EVENTS
(Yield in Kilotons)

Year	Total Fission Yield	Total Fission Yield from Events, the Total Yield of Which Was 1 MT or Greater
1945	60	
1946	40	
1948	100	
1951	500	
1952–1954	37,000	36,000
1955	200	
1956	9,000	8,000
1957–1958	19,000	14,000

TABLE 3

SOVIET NUCLEAR EVENTS
(Yield in Kilotons)

Inclusive	Total Fission Yield*
1945–1951	60
1952–1954	500
1955–1956	4,000
1957–1958	21,000

TABLE 4

UNITED STATES, UNITED KINGDOM, AND SOVIET NUCLEAR EVENTS
(Yield in Kilotons)

	Fission Yield*			Total Yield	
Inclusive Years	Air Burst	Ground Surface Burst	Water Surface Burst	Air Burst	Surface Burst
1945–1951	190	550	20	190	570
1952–1954	1,000	15,000	22,000	1,000	59,000
1955–1956	5,600	1,500	6,000	11,000	17,000
1957–1958	31,000	4,400	4,600	57,000	28,000

* A value of 50% has been arbitrarily selected for the fission to total yield ratio for all Soviet thermonuclear tests. As indicated in the tables, 50% is about the average fission to total yield ratio for all U.S./U.K. thermonuclear tests.

Anticipating for the moment the result for which we present evidence presently, namely that Martell's theory is essentially correct, one then should subtract from the 11 megatons excess in the spring of 1958 in the Northern Hemisphere approximately 3 megatons estimated tropospheric fallout from the Northern Latitude test sites to find an excess of some 8 megatons from Russian Polar debris. Now having set aside some 8 megatons of Russian Polar stratospheric injection from the general worldwide stratospheric pool of 26 megatons given in Figure 3, the rate of uniform worldwide fallout observed in the years between 1956 and 1958, 2.2 megatons per year in the Southern Hemisphere on the average, is to be compared with 18 megatons and in this way we calculate that the stratospheric residence time for equatorial injections is between 4 and 5 years, corresponding to a half-life of about 3 years on the average.

In order to study the general rate of spread of the Russian October debris we present in Figure 2 the data of the Naval Research Laboratory (L. B. Lockhart[31]) on the concentration of radioactive fallout mixed fission products at a variety of positions along the eightieth western meridian, for the months in the late summer and early fall of last year when the Pacific Operations were being conducted and terminated and in Figure 4 the analogous results for November, 1958 through

February, 1959 showing the fallout from the Russian October test series. The data in Figure 2 indicate that the equatorial material spreads into both hemispheres in a roughly symmetrical manner. Thus we see that the stratospheric content of equatorially injected fission products in the Southern Hemisphere should not be too greatly different from that in the Northern Hemisphere. Figure 4, on the other hand, shows clearly that this does not happen for Polar debris in the first four

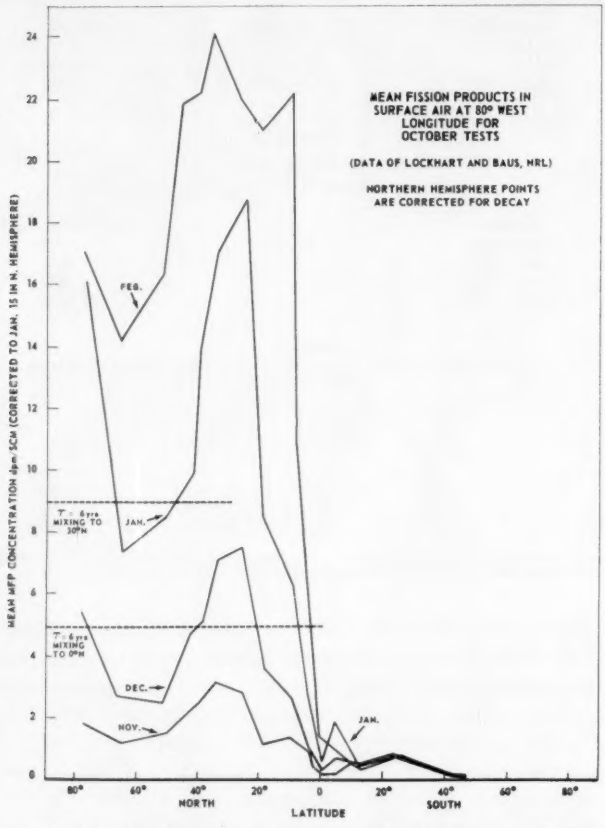
MEAN FISSION PRODUCTS IN SURFACE AIR AT 80° WEST LONGITUDE FOR OCTOBER TESTS
(DATA OF LOCKHART AND BAUS, NRL)
NORTHERN HEMISPHERE POINTS ARE CORRECTED FOR DECAY
MEAN MFP CONCENTRATION dpm SCM (CORRECTED TO JAN. 15 IN N. HEMISPHERE)
FEB.
JAN.
DEC.
NOV.
JAN.
T = 6 yrs MIXING TO 30MM
T = 6 yrs MIXING TO 0MM
NORTH
SOUTH
LATITUDE

FIG. 4.—Mean fission product content of surface air following Russian October 1958 test series.

months at least, but that mixing seems to have occurred as far south as the equator and to have essentially stopped there up to that time.

Figure 5 shows the data for the stratospheric content of strontium-90 as taken between 50,000 and 90,000 feet altitude in the period November, 1956 to November, 1958 by the ASHCAN balloon borne air filter project. The average concentrations in the 50,000 foot to 90,000 foot altitudes range were 24 ± 2.2 dpm/1,000 SCP at Sao Paulo, Brazil, and 26 ± 2.5 at Minneapolis and 32 ± 4.3 at San Angelo, Texas. From these it seems that the Southern Hemisphere stratosphere may not have been

appreciably lower than the Northern Hemisphere in this period. This conclusion must be taken with reserve, however, since the data themselves show poor internal consistency as regards the Cs^{137}/Sr^{90} ratio and because of the fact that there is only one station in the Southern Hemisphere.

In order to observe the fallout from the Russian October series promptly and with adequate accuracy, the standard procedure of strontium-90 fallout analysis was changed to allow up-to-date analyses of rainfall occurring during the extremely

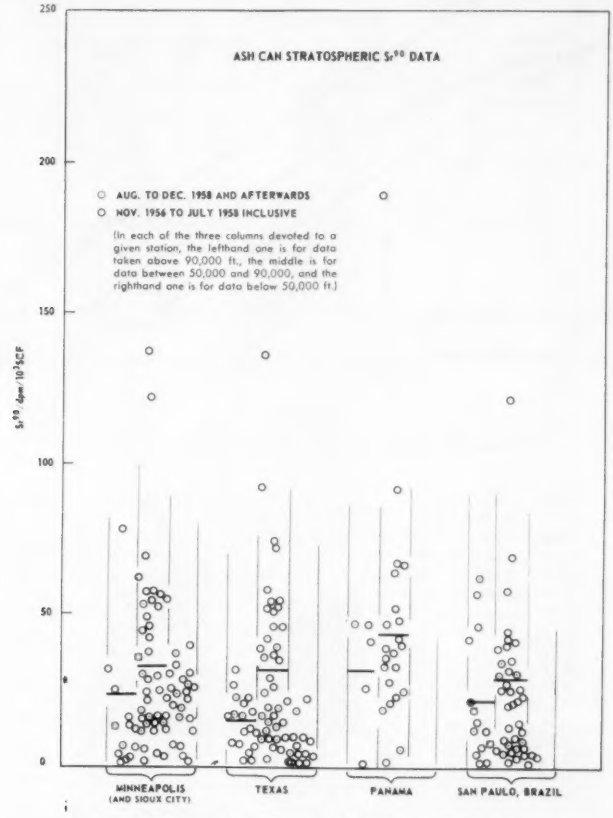
ASH CAN STRATOSPHERIC Sr90 DATA
AUG. TO DEC. 1958 AND AFTERWARDS
NOV. 1956 TO JULY 1958 INCLUSIVE
(In each of the three columns devoted to a given station, the lefthand one is for data taken above 90,000 ft., the middle is for data between 50,000 and 90,000, and the righthand one is for data below 50,000 ft.)
250
200
150
100
50
0
MINNEAPOLIS (AND SIOUX CITY)
TEXAS
PANAMA
SAN PAULO, BRAZIL

FIG. 5.—Strontium-90 content of stratospheric air.

crucial periods of March, April, and May of 1959. The change consisted in a reliance on the smoothness of the curve of the ratio Sr^{89}/Sr^{90} (Fig. 6) versus time in view of the fact that no nuclear explosions have been fired recently and the use of the knowledge of this ratio by extrapolation of the curve to allow one to calculate the strontium-90 fallout from a measurement of the total radioactive strontium ($Sr^{89} + Sr^{90}$) in a rainfall sample. This measurement can be made quickly. In this way the data in Figure 7 for rain collected at the Geophysical Laboratory in Washington, D.C. have been obtained and are given in detail in Table 5. They

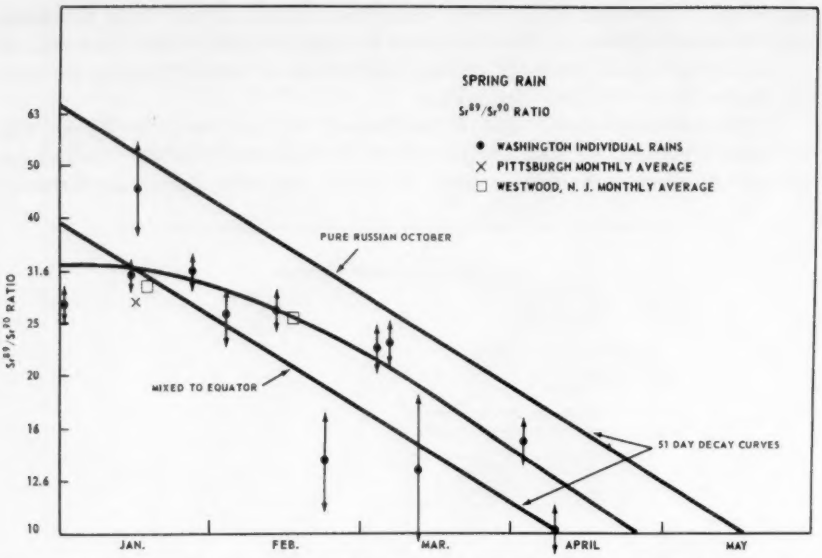

FIG. 6.—Ratio of strontium-89 to strontium-90 in rainwater samples. The samples were collected in the spring of 1959.

TABLE 5

SUMMARY OF WASHINGTON RUSSIAN OCTOBER DATA

Date	Rainfall	Sr^{89} dpm/l on Date	Sr^{90} dpm/l	Sr^{89}/Sr^{90}	Sr^{90} Rate $\mu c/mi^2/in.$	Incremental Sr^{90} $\mu c/mi^2$	Total Sr^{90} mc/mi^2
Jan. 1	0.88	203 ± 1.7	7.4 ± 0.4	27.6 ± 1.7	222 ± 14	196 ± 11	0.20
Jan. 14–15	0.12	890 ± 9	28.6 ± 1.3	31.2 ± 2	860 ± 37	104 ± 5	0.30
Jan. 15–16	0.34	2,230 ± 65	48.6 ± 9.3	45.9 ± 9	1,460 ± 280	496 ± 96	0.80
Jan. 20	0.33	639 ± 55		(31)		(202)	1.00
Jan. 21–22	0.42			(31)		(202)	1.20
Jan. 26–27	0.76	1,450 ± 13	45 ± 2	31.8 ± 1.9	1,365 ± 60	1,040 ± 46	2.24
Feb. 3–4	0.43	259 ± 3	10.1 ± 1.1	25.7 ± 3	303 ± 33	131 ± 14	2.37
Feb. 12–14	0.75	722 ± 15	27.1 ± 1.7	26.7 ± 2	815 ± 51	611 ± 28	2.98
Feb. 23	0.18	1,085 ± 40	80 ± 14	13.9 ± 3	2,140 ± 420	434 ± 76	3.42
Mar. 4–5	Trace	950 ± 40	42 ± 4	22.6 ± 2.4	1,260 ± 126		
Mar. 5–6	1.13	724 ± 203	32.4 ± 2.6	23 ± 2	974 ± 78	1,104 ± 88	4.52
Mar. 11–12	0.52	446 ± 25	35 ± 13	13 ± 5	1,050 ± 390	544 ± 203	5.06
Mar. 27	0.44	580 ± 30		(13)		(585)	5.65
Mar. 30	0.30	636 ± 36		(13)		(436)	6.09
Apr. 2 6 A.M. to 1 P.M.	0.29	276 ± 50	23.4 ± 2	15.3 ± 1.3	700 ± 60	519 ± 44	6.60
Apr. 2 1 P.M. to 5 P.M.	0.45	397 ± 17					
Apr. 3–4	0.69	236 ± 16		(13)		(378)	6.98
Apr. 9–10	0.31	550 ± 50	51.5 ± 3.7	10 ± 1	1,545 ± 110	479 ± 35	7.46
Apr. 10–12	1.17	232 ± 13		(12)		(688)	8.15
Apr. 19–20	0.12	1,337 ± 27		(11)		(440)	8.59
Apr. 27–28	0.65	553 ± 55		(10)		(1000)	9.59
Apr. 28–29	0.48	380 ± 20	50.5 ± 15	7.6 ± 2.3		730 ± 216	10.32
May 2–3	0.11					(150)	10.47
May 12–13	0.12					(180)	10.65
May 13	1.05	120 ± 5		(8)		(430)	11.08

The numbers in parenthesis are derived by taking the curve as drawn in Figure 6, calculating from it the expected strontium-89/strontium-90 ratio and using the observed total strontium activity in the sample of rainfall involved to calculate the strontium-90 contribution, and thus to calculate the total fallout in the storm in question.

show an average fallout rate over the last five months of about 2.3 millicuries of strontium-90 per square mile per month, a rate which can only be explained by the Russian October fallout coming down with about a 1-year residence time as Dr. Martell had suggested. Data from Bedford, Massachusetts,[10] Pittsburgh, Pennsylvania,[11] and Westwood, New Jersey,[12] are included in the figure to show the

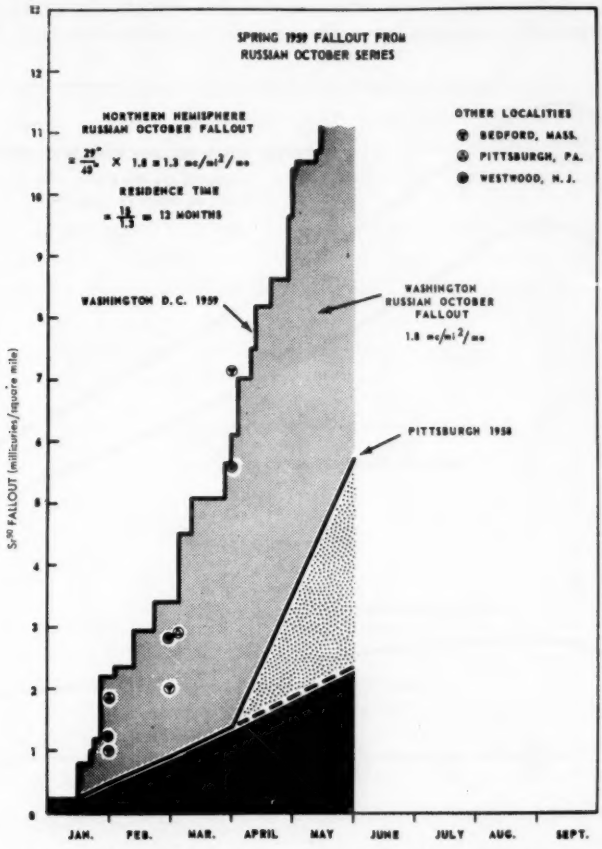

FIG. 7.—Fallout in the spring of 1959 attributed to the Russian October 1958 tests. This chart is based primarily on rainwater samples collected in Washington, D. C., in the spring of 1959. Since corresponding samples had not been collected in Washington in 1958 it was necessary to base the correction curve on 1959 samples from Pittsburgh. After allowing for the difference in rainfall at the two sites, the 1959 data for Pittsburgh are expected to agree with the corresponding Washington data.

general agreement. The mean residence time is calculated, as shown in Figure 7, by subtracting the 1958 rate for Pittsburgh after removing the April, 1958 rise which it showed, presumably due to the Russian tests at the end of February, 1958, and multiplying by the ratio of the mean annual rainfall to that for Washington. Thus we see that there is a difference in residence time for polar and equatorial shots

injecting into the stratosphere and we consequently are led to speculate as to its cause and as to whether the residence time for intermediate latitudes would be intermediate between 1 and 5 years.

The question of the Machta theory of extra rapid stratospheric fallout rates in the middle latitudes remains not completely settled in the author's opinion. However, one point is certainly clear from the soil data seen in Figure 1: the rate of fallout in equatorial latitudes is less than it is in latitudes away from the equator

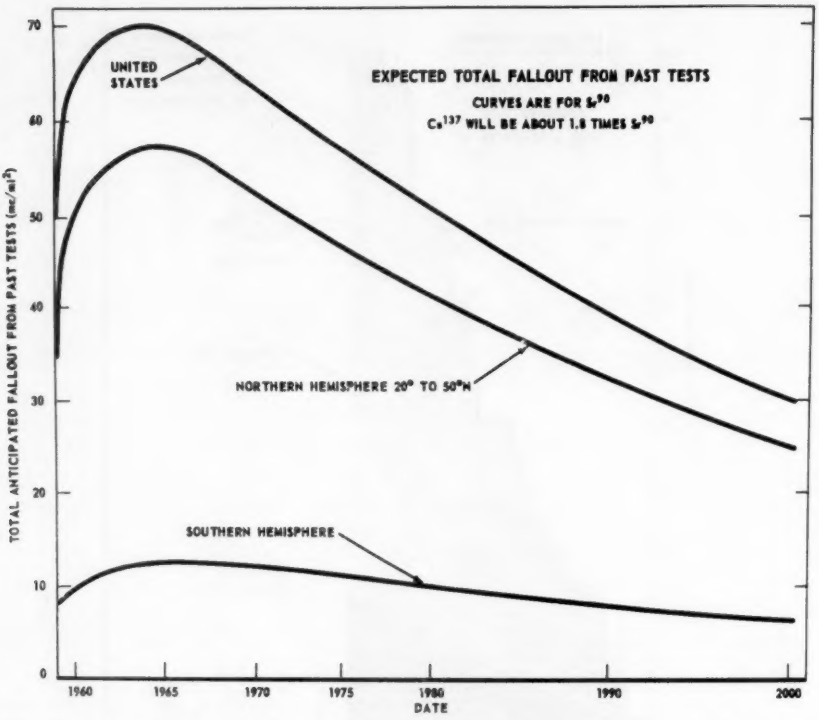

FIG. 8.—Fallout levels predicted on the basis of no further tests. These curves are based on Table 6.

and this is an essential point of Machta-Brewer theory. So it would seem that this feature is correct. Also, the short 1-year residence time for polar injections is in general in keeping with the Machta-Brewer Model of rising stratospheric air in equatorial regions and falling stratospheric air elsewhere, particularly in Polar regions.

Future Fallout Expected from Past Tests.—Using the results of Table 1 showing the U.S. to have about 15 mc/mi² more strontium-90 than equivalent latitudes in other countries and taking the latitudinal profile in these foreign countries as roughly characteristic of Polar stratospheric fallout even though we believe some 3 megatons equivalent of the total excess of 11 in the Northern Hemisphere as of the summer of

1958 had tropospheric origin, we can predict the future fallout to be expected from past tests.

Neglecting for the moment the past and taking only the future beginning January 1, 1959, there are two terms—the Russian October and the worldwide reservoir. Then the total future deposition in the Northern Hemisphere t years after January 1, 1959 would be expected to be

$$[9(1 - e^{-t/5}) + (12.5 \text{ to } 15)(1 - e^{-t})]e^{-t/40}$$

in megatons fission equivalent (or in millicuries of strontium-90 per square mile if spread uniformly), the first term being the uniform stratospheric fallout with a residence time of 5 years and the second being the Russian October with a mean residence time of 1 year. The final factor is for the radioactive decay of the strontium-90.

For any given latitudinal belt the prediction is obtained by using the soil data profile of Figure 1 and adding for the Nevada extra contribution for the U.S. Thus we obtain the results shown in Figure 8 and Table 6. Since the soil data in

TABLE 6

PREDICTED FUTURE FALLOUT STRONTIUM-90 LEVELS FROM PAST TESTS
(millicuries per square mile)

Year	Average U.S.	Average, Foreign 20°N to 50°N	Average, Southern Hemisphere
1959	50	35	8
1960	64	50	10
1965	70	57	13
1970	64	52	12
1975	57	47	11
1980	51	42	10
1990	39	33	8
2000	31	25	6

Figure 1 apply to samples taken in the early months of 1958, a correction derived from the rain pot data is made to January 1, 1959. This brings the worldwide fallout (exclusive of the U.S. excess) up to about 30 megatons.

For example, the U.S. average future strontium-90 total fallout in mc/mi² should be obtained from

$$F(t) = [15 + 35 + 9 \times (1 - e^{-t/5}) + (12.5 \text{ to } 15) \times 22/11(1 - e^{-t})]e^{-t/40}$$

In this, the first number 15 is the average U.S. excess from Table 1, the second is the estimated mean in the 20° to 50°N latitude band as of January 1, 1959. The third term is the worldwide fallout using only the 9 megatons that will be expected in the Northern Hemisphere and the fourth is the Russian October term. The asymmetry factor of 22/11 is taken from Figure 1 as the ratio of the average observed fallout in the 20° to 50° band, less the 7 mc/mi² of uniform worldwide, to the hemisphere-wide average of 18 mc/mi² less the 7 mc/mi² also, or 11 mc/mi².

Bomb Carbon-14 and Tritium.—As pointed out earlier,[5] the neutrons escaping from a nuclear device during a nuclear explosion in air will make carbon-14 almost quantitatively just as the cosmic ray produced neutrons do in the genesis of natural radiocarbon. As stated previously, as of January 1, 1958, an estimated 10^{28} carbon-14 atoms had been injected into the atmosphere, mostly in the stratosphere. The present total figure should be higher in approximate proportion to the total

estimated yields. This is to be compared with a normal stratospheric carbon-14 content of about 9×10^{27} carbon-14 atoms so the stratospheric carbon-14 content should have been about doubled.

Data on the rise in carbon-14 content of the troposphere and living matter are given in Figures 9 and 10 and the recent literature.[4, 13–19] From these it is clear that the carbon-14 descent from the stratosphere is not out of keeping with a residence time of several years, possibly five, although our knowledge of the stratospheric reservoir content is imperfect.

The similarity between the rises observed in the Northern and Southern Hemispheres suggest a short latitudinal mixing time of the order of two years or less as pointed out by Fergusson[18] with the Suess fossil fuel CO_2 effect, and Broecker and

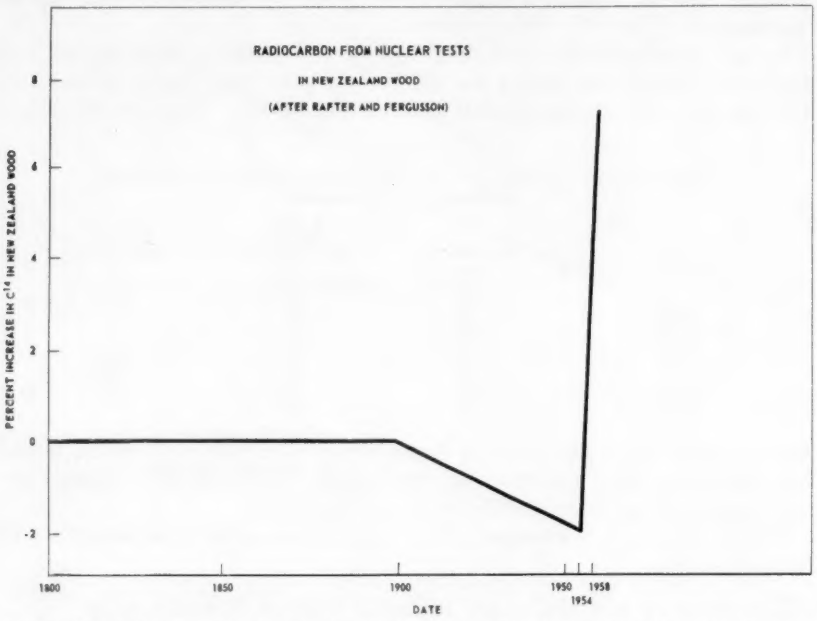

FIG. 9.—Effect of radiocarbon from nuclear tests on the radiocarbon content of wood. Decrease prior to 1954 is attributed to combustion of fossil fuels; increase after 1954 to nuclear tests.

Walton[14] with the bomb carbon-14. The estimates of the amount by which bomb carbon-14 will raise the general carbon-14 level when complete mixing with the oceans has occurred has risen from 0.3 to about 0.4 or 0.5 per cent now due to the firings during 1958.

Of course, in the short time range before the deep ocean mixes, the concentration of radiocarbon in the biosphere will rise as the stratospheric material descends. If the total exchangeable carbon reservoir in the top layer of the ocean and the troposphere and biosphere is X times that in the stratosphere and the present carbon-14 increases in the stratosphere is, say 200 per cent, then we must expect 200/X per cent as the eventual increase in the troposphere, biosphere, and top ocean. Since X is about 14 we should then expect an increase of about 14 per cent

which would last for several years. If the mixing with the top ocean is slow relative to the mixing of the atmosphere there may be a rise above this value for a few years after which it will settle back and gradually, over a matter of several centuries, fall to the 0.4 to 0.5 per cent value, depending on the time required to mix with the deep ocean. Subsequent to this it will decay with its 5,600 year half-life.

The movement of the bomb carbon-14 should help considerably in solving the remaining mysteries of atmospheric mixing patterns and storage times.

Several points about the biological effects of this carbon-14 have been made in recent publications.[20–22]

As has been remarked previously, all of the hydrogen bombs release a considerable

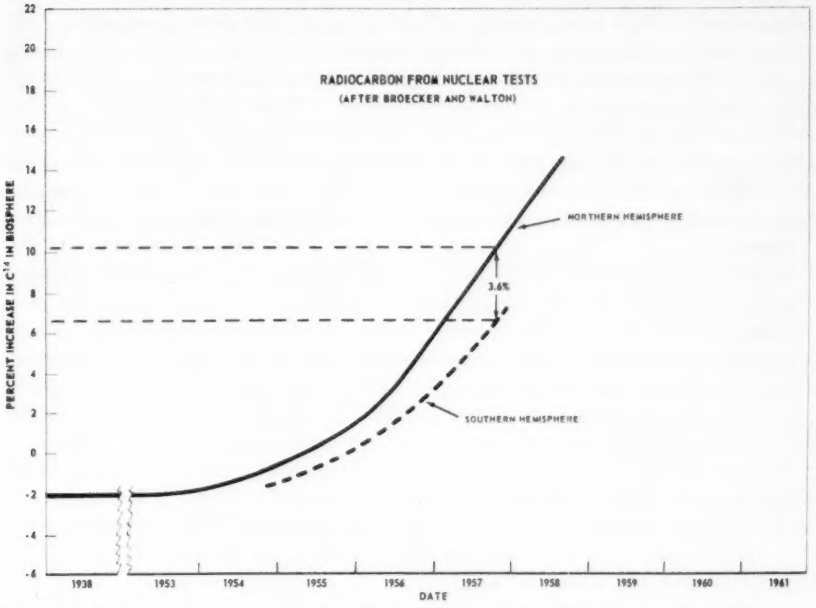

FIG. 10.—Effect of radiocarbon from nuclear tests on the radiocarbon content of the biosphere.

amount of tritium but because the devices previously have always been fired in the troposphere only a part of the radioactive water formed from this tritium has remained in the stratosphere. This is because a large quantity of water is incorporated in the fireball under these conditions with the result that when the fireball rises into the stratosphere and cools it makes the familiar white cloud consisting of rather large ice crystals which fall rather quickly and thus carry a considerable part of the tritium back to the troposphere again. This did not happen for the two devices fired over Johnson Island in the stratosphere in August, 1958 and, therefore, we can expect a relatively large increase at this time in the stratospheric tritium water content over that the cosmic rays have put into the stratosphere *naturally*. The quantities produced by the bombs could well raise this *natural* level several hundred fold.

The stratosphere is so low in moisture (a fair estimate seems to be about 10 milligrams per square centimeter of the earth's surface for the entire stratosphere as compared to something like 2 to 3 grams per square centimeter for the troposphere) that the concentration of the cosmic ray tritium in the stratospheric moisture should be very high, something like in the inverse ratio of the water concentrations, together with an additional factor for the 5 year storage time of the cosmic ray produced tritium.

In substance, the hydrogen bombs fired over Johnson Island made tritium water in large quantities in the stratosphere and it is to be expected if the simple uniform model for equatorial injections is correct, that the rains all over the world will show a stratospheric tritium drip as well as the stratospheric strontium-90-cesium-137 fission product drips which have been occurring for years. But this stratospheric tritium drip probably will have had its zero time in August of 1958 since earlier tropospheric firings probably added relatively less. This tritium should be useful in studying the normal hydrological cycles of the atmosphere and hydrosphere and the circulation patterns of the oceans. So it is with real interest that we look forward to the next few months of rainfall to determine the mechanism by which moisture in the top part of the world's atmosphere mixes with the lower stratosphere and with the troposphere and the land and ocean waters. This may prove to be an important contribution to meteorology and geophysics if it develops as expected.

A further point is that the Russian October shots having been fired in cold dry Polar air may have left more in the stratosphere than other tropospheric explosions and this coupled with the shorter residence time of the Martell theory should add appreciably to the Johnson Island tritium water fallout in the Northern Hemisphere.

A crude theory based on the assumption that about half of the water in the ice crystals in the equatorial stratospheric clouds evaporates before the crystals fall down into the troposphere and using a yield of tritium proportional to the fusion yields from Tables 2, 3, and 4, and assuming Martell's theory is correct for Polar shots, predicts a value of 800 to 1,000 tritium units (10^{-18} T's per H atom) for rain in the Northern Hemisphere in the spring and summer of 1959. This, though a level entirely safe from a health standpoint, makes the measurement of tritium contained in rain and surface water at such levels relatively simple.

* The total water in the stratosphere is about 0.01 gm/cm² or less, while that in the troposphere is about 2 gm/cm², 200 times as much.

† It is useful to note that 1 megaton fission spread uniformly worldwide gives $^1/_2$ mc Sr^{90}/mi² or if restricted to one hemisphere and spread uniformly gives 1 mc/mi².

[1] Martell, E. A., "Atmospheric Aspects of Strontium-90 Fallout," *Science*, **129**, 1197–1206 (1959).

[2] Greenfield, S. M., "Rain Scavenging of Radioactive Particulate Matter from the Atmosphere," *Journal of Meterology*, **14**, 115–125 (1955).

[3] Hardy, Edward P., Jr., John Harley and S. Allan Lough, "Environmental Contamination from Weapons Tests." U.S. Data Summarized. HASL-42. AEC Health and Safety Laboratory, New York. There are two supplements: HASL-51 (Nov., 1958) and HASL-55 (Feb., 1959). All three are available at Technical Information Services, Department of Commerce, Washington, D.C.

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*GENETIC RECOMBINATION BETWEEN ESCHERICHIA COLI AND SALMONELLA TYPHIMURIUM**

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Introduction.—The phenomenon of genetic recombination by sexual mating in bacteria, described by Tatum and Lederberg[1] has been investigated intensively by Lederberg *et al.*[2] and many other workers. These classical studies were carried out almost entirely with the K-12 strain of *Escherichia coli*, although comparable results were obtained with a small number of other strains of *E. coli*.[3,4]

In further studies, Cavalli[5] found that the frequency of recombination could be greatly increased by using a highly fertile mutant of the K-12 strain. Additional high frequency of recombination (Hfr) strains of K-12 have been isolated in a number of laboratories.[6,7] The concept of compatibility among certain K-12 derivatives has led to a detailed description of the fertility factor (F), so that it is now understood that mating consists of a unilateral contribution of genetic material from the Hfr or the F^+ strain to the recipient Hfr, F^- or F^+ organism.[8–11]

In an attempt to demonstrate recombination between diverse species of bacteria, Zinder and Lederberg[12] discovered another mode of genetic transfer involving bacteriophage particles as vectors of genetic material. Intensive investigation of this phenomenon, referred to as genetic transduction, was undertaken by Stocker *et al.*,[13] Lederberg and Edwards,[14] and Zinder,[15] using the phage PLT-22. Studies of additional bacterial viruses competent in transduction have been reported by Baron *et al.*,[16] Spilman *et al.*,[17] Sakai and Iseki,[18] Lennox,[19] and Morse *et al.*[20] Many of these results have dealt with the phage-mediated transfer of genetic material between different species of bacteria and even organisms classified in different genera have been altered by a suitable phage.[19]

Genetic recombination between bacteria of different genera, however, has only recently been reported by Luria and Burrous[21] with the demonstration of mating between *E. coli* K-12 and various species of Shigella. The results of these workers have indicated the presence of the same fertility system in Shigella species as is known to exist in the *E. coli* mating strains. Attempts at recombination of *E. coli* with Salmonella species by a number of workers, however, have been uniformly negative.

It is the purpose of this communication to describe experiments which involve the genetic recombination of *E. coli* and *Salmonella typhimurium* at high frequency. Certain of the results which have been obtained more recently suggest a generalized ability of other Salmonella species to mate with Hfr strain of *E. coli* at a much lower frequency. Preliminary data concerning this ability in other Salmonella species have been reported by Baron *et al.*[22] and these results will be detailed in a future communication.

Materials and Methods.—Cultures: A number of previously described derivatives of *E. coli* strain K-12 were employed; W1895 (Hfr), W-6 (F^+), and W1485 were obtained from Dr. P. D. Skaar.

S. typhimurium strain TM-9 was from the culture collection of the Walter Reed Army Institute of Research, as were other Salmonella species employed in some experiments. These strains were originally obtained from Dr. P. R. Edwards.

Isolation of streptomycin resistant mutants (S^r) was accomplished by plating dense suspensions of streptomycin sensitive cells (S^s) on meat extract agar containing 600 micrograms per ml of streptomycin sulfate (Abbott or Pfizer).

Phages: The T series of coliphages was obtained from Dr. G. Bertani and were propagated on *E. coli* B and other host strains; phage PLT-22 was received from Dr. J. Lederberg and propagated on a number of suitable host strains of Salmonella. Phage sensitivity was checked by cross-streaking the phage preparations with the bacterial suspension to be tested on meat extract agar.

Media: Meat extract agar was made to contain: Meat extract 3 g, peptone 10 g, NaCl 5 g, agar 20 g, and distilled water 1,000 ml.

Minimal medium consisted of a separately autoclaved Difco Noble agar base made up double strength (14 g/400 ml water), to which was added 300 ml of physiological saline, 40 ml of a solution of KH_2PO_4 (60 g/l) and K_2HPO_4 (140 g/l), 40 ml of a solution of $(NH_4)_2SO_4$ (20 g/l) and $MgSO_4\ 7H_2O$ (2 g/l), and 10 ml of a 20 per cent solution of the appropriate carbohydrate. The carbohydrates employed (lactose, *l*-arabinose, *l*-rhamnose, etc.) were Pfanstiehl products which were sterilized by filtration.

Eosin-Methylene Blue agar was made following the usual formula (Difco), but excluding lactose; in addition, the Baltimore Biological Laboratories kindly supplied a preparation of EMB agar which did not contain lactose. Where required, 20 ml of a 20 per cent solution of the appropriate sugar was added to each liter of this medium. Minimal EMB agar was prepared by the addition of the necessary amounts of the dyes to the minimal medium described above. EMB streptomycin agar contained 600 micrograms of streptomycin sulfate per ml.

The Difco products, Penassay broth, Nutrient agar, Bacto-agar, and Phenol red broth were employed for routine cultivation, transfer, and testing of the strains.

Antisera: Sera to *E. coli*, Salmonella species, and recombinants were prepared in rabbits by a series of injections of approximately 10^9 living cells of these organisms, or by injections of suspensions of organisms which were heat-killed at 100 C for two hours. Shigella hybrid F22 (from Shigella x coli) and antiserum to this strain[21] were very kindly supplied by Dr. S. E. Luria.

Results.—A number of species of Salmonella were tested for their ability to mate with the Hfr or the F^+ strain of *E. coli* K-12 in preliminary experiments. The species of Salmonella selected consisted of representative serotypes typical of

the various groups of the Kaufmann-White diagnostic scheme. No attempt was made at this time to undertake a complete screening of the approximately four hundred or more characterized serotypes of the genus Salmonella, or of the huge number of isolates of any one serotype which were available.

The cultures of *E. coli* and Salmonella were grown together in Penassay broth for approximately 24 hours at 37 C without aeration, centrifuged and washed three times with physiological saline, and then plated on minimal agar plates containing lactose (Lac) as the sole carbon source. The *E. coli* Hfr and F^+ strains require methionine (M^-) for growth, while an important criterion for classification of organisms in the genus Salmonella is their inability to ferment or utilize lactose. The minimal lactose (ML) agar medium was employed since it would fail to support the growth of either parental mating strain, but could detect M^+ Lac^+ progeny. For providing a control, the organisms also were grown separately; all the strains tested were stable lactose negatives (Lac^-) and reversions to methionine independence by the *E. coli* strains were observed only rarely in control platings.

In the initial experiment, a smooth culture of *S. typhimurium* strain TM-9, with the antigenic structure IV V XII:i,1,2 mated with the Hfr strain of *E. coli* at low frequency; recombination of this strain could *not* be detected with the *E. coli* F^+ strain. The *E. coli* Hfr would represent the best available strain for demonstration of lactose positive (Lac^+) recombinants since it has been characterized by its high frequency ability for the lactose factor.[5] Failure to observe recombination using the F^+ strain is to be expected when the frequency of mating with the Hfr strain is low. The colonies which appeared on the ML plates in the mating with the *E. coli* Hfr were examined and found to be Lac^+ forms exhibiting the antigenic characteristics of the *S. typhimurium* parent strain.

In further experiments, the plating procedure of mixing washed suspensions of the mating strains directly on ML agar or other selective media as described by Luria and Burrous[21] was used. In agreement with their observations, frequencies of recombination were only slightly higher when parent strains were incubated together for a short time prior to plating.

The frequency of recombination of *S. typhimurium* strain TM-9 with the *E. coli* Hfr strain (expressed as the ratio of recombinants to the number of Hfr parent cells) was approximately 4×10^{-8} using the procedure of mixing the strains directly on ML agar. About 2×10^9 cells of the recipient parent strain were employed usually. Frequencies of this low order have been observed with a number of other species of Salmonella by Baron *et al.*[22]

For purposes of convenience in selection and purification of recombinants, streptomycin-resistant variants of strain TM-9 were obtained as described earlier. Individual streptomycin-resistant clones (S^r) were tested for their ability to cross with the Hfr strain of *E. coli* on minimal lactose and on EMB agar, both containing 600 micrograms of streptomycin per ml. Five S^r clones checked in this experiment were capable of crossing with the Hfr strain at a greatly increased frequency, while one S^r colony obtained from the same plate of streptomycin agar continued to recombine at the low frequency characteristic of the original strain. For further studies, an S^r isolate, referred to as *S. typhimurium* strain TM-9S^r-2, was selected which showed a recombination frequency of 1×10^{-4} when plated on selective media with or without streptomycin. It was also now possible to detect recom-

binants of this TM-9S^r-2 strain by mating it with the F$^+$ strain of *E. coli*. Table 1

TABLE 1

FREQUENCY OF RECOMBINATION OF *Salmonella Typhimurium* STRAINS (SELECTION FOR Lac$^+$ HYBRIDS)

Recipient	*E. coli* Hfr	*E. coli* F$^+$
S. typhimurium 9	4×10^{-8}	0
S. typhimurium 9S^r-2	1×10^{-4}	2.8×10^{-6}
S. typhimurium LT-7	1×10^{-8}	0
S. typhimurium HB	1×10^{-8}	0

depicts the frequencies of recombination of several strains of *S. typhimurium* mated with Hfr and F$^+$ *E. coli*.

A number of subsequent attempts were made to determine whether streptomycin resistance was correlated in some measure with the ability of certain S^r clones to recombine at this higher frequency. A higher rate of recombination could be considered as the frequency typical of a true F$^-$ strain, or could be due to an increase in mutation frequency to the F$^-$ state in certain strains. Although other hypotheses are possible, the assumption was made that strains of *S. typhimurium* are F° (unable to act as recipients), but can mutate at low frequency to the F$^-$ state and that it was these occasional F$^-$ cells in the population which recombined with the Hfr *E. coli*. This assumption bears a similarity to the concept proposed by Jacob and Wollman[6] that only those F$^+$ *E. coli* cells which mutate to or in some manner acquire the Hfr state are capable of mating as donors. The failure to detect recombinants from an *E. coli* F$^+$ mating of strain TM-9 can easily be explained on the basis that it is extremely unlikely that an appropriate cell of *S. typhimurium* (one becoming F$^-$ from the F° state) would encounter an *E. coli* F$^+$ cell which has acquired Hfr ability.

The fortuitous association of F$^-$ strains (or at least strains with an enhanced ability to acquire the F$^-$ condition) with the S^r property in some clones of *S. typhimurium* was indicated when additional S^r clones were examined in further experiments. Approximately fifty individual S^r mutants were studied and these were essentially similar in behavior to the parent *S. typhimurium* strain TM-9 with regard to recombination ability. In addition, S^r mutants of other strains of *S. typhimurium* and of various other Salmonella have been tested without the isolation of other high frequency recipient strains, although slight increases in the ability of certain species to recombine have been noticed.

This approach was investigated in the hope that it might lead to a rational method for the acquisition of the more useful F$^-$ mutants which evidently can occur in many species of Salmonella. Since the Lac$^+$ recombinants (strain TM-9 × coli Hfr) were uniformly streptomycin sensitive, that does not support any causal association or correlation between resistance to streptomycin and fertility. The limited studies undertaken with the S^r mutants, however, do not rule out the possible existence of linkage involving the streptomycin locus and a determinant controlling recipient ability. This possibility can best be established with a more comprehensive examination by chromosomal mapping methods of suitable material.

Genetic examination of recombinants: A strain of *S. typhimurium* (TM-9S^r-2) was now available with a high enough frequency of recombination to allow for the convenient testing of other genetic changes, namely, the appearance and frequency

of various unselected markers. In order to obtain further factors for linkage analysis, the TM-9S^r-2 strain was treated with ultraviolet light and plated on EMB agar containing various sugars. In this manner, an *l*-arabinose negative (Ara^-) and an *l*-rhamnose negative (Rha^-) derivative of this culture was isolated, factors which could be studied as both selected and unselected markers. The characteristics of the parent strains employed in these preliminary attempts to establish linkage relationships are listed in Table 2, as are the selected and unselected markers available for testing in the hybrids. Table 3 provides data determined from the frequency of the unselected markers which were transferred from the *E. coli* parent to the Salmonella hybrids (selection for lactose, arabinose, or rhamnose utilization).

TABLE 2

CHARACTERISTICS OF PARENT STRAINS AND SELECTED AND UNSELECTED MARKERS

Salmonella parent	*E. coli* Hfr parent Lac^+ $Inos^-$ Rha^+ Ara^+ Ind^+ S^s M$^-$ Dul^- λ^+	
S. typhimurium TM-9Sr-2	Selected	Unselected
Lac^- $Inos^+$ Rha^+ Ara^+ Dul^+ Ind^- S^r λ^r	Lac^+	Dul^-, $Inos^-$, Ind^+, λ^+, S^s
Lac^- $Inos^+$ Rha^+ Ara^- Ind^- S^r λ^r	Ara^+	Lac^+, $Inos^-$, Ind^+, λ^+, S^s
Lac^- $Inos^+$ Rha^- Ara^+ Ind^- S^r λ^r	Rha^+	Lac^+, $Inos^-$, Ind^+, λ^+, S^s

Key: Lac = lactose, Ara = arabinose, Dul = dulcitol, Inos = inositol, Ind = indol, M = methionine, Rha = rhamnose, S = streptomycin, and λ = phage lambda.

TABLE 3

CLASSES AND FREQUENCY OF HYBRID COMBINATIONS OF *S. typhimurium*

Hybrid Classes—Selection for Lac^+	No. of Hybrids	Frequency, %
Rac^+ $Inos^+$ Dul^+ Ind^- λ^r S^r	136	90.6
Lac^+ $Inos^+$ Dul^- Ind^- λ^r S^r	8	5.3
Lac^+ $Inos^-$ Dul^+ Ind^- λ^r S^r	6	4.0
Hybrid Classes—Selection for Ara^+		
Ara^+ $Inos^+$ Lac^+ Ind^- λ^r S^r	91	60.3
Ara^+ $Inos^+$ Lac^- Ind^- λ^r S^r	12	7.9
Ara^+ $Inos^-$ Lac^+ Ind^- λ^r S^r	12	7.9
Ara^+ $Inos^-$ Lac^- Ind^- λ^r S^r	36	23.8
Hybrid Classes—Selection for Rha^+		
Rha^+ Lac^+ $Inos^+$ Ind^+ λ^r S^r	2	1.0
Rha^+ Lac^+ $Inos^+$ Ind^- λ^r S^r	4	2.1
Rha^+ Lac^+ $Inos^-$ Ind^- λ^r S^r	8	4.2
Rha^+ Lac^+ $Inos^-$ Ind^+ λ^r S^r	0	0
Rha^+ Lac^- $Inos^-$ Ind^- λ^r S^r	86	44.8
Rha^+ Lac^- $Inos^-$ Ind^+ λ^r S^r	8	4.2
Rha^+ Lac^- $Inos^+$ Ind^- λ^r S^r	78	40.6
Rha^+ Lac^- $Inos^+$ Ind^+ λ^r S^r	6	3.1

In these experiments, the recombinants which appeared on the minimal agar plates were purified by streaking on EMB streptomycin agar plates and single colonies were selected for further study. The results listed each represent the analysis of between one to two hundred colonies selected and purified in this manner. Carbohydrate fermentation reactions were determined by the inoculation of cell suspensions of the hybrids into tubes of phenol red broth containing 1 per cent of the appropriate carbohydrate.

The results of selective platings demonstrated that the frequency of recombination was highest when selecting for Lac^+ recombinants, lower for Ara^+ recombinants, and lowest for Rha^+ recombinants ($Lac^+ > Ara^+ > Rha^+$). In addition, from the data in Table 3, it can be concluded that the locus for *l*-arabinose utilization must be linked to the lactose locus, since in the mating with *l*-arabinose as

the selective marker, a large percentage of the recombinants which appeared were also Lac$^+$. It was possible also to demonstrate the presence of recombinants capable of producing indol, but only following the selection of Rha$^+$ recombinants. The introduction of the loss of a fermentative ability, the utilization of inositol was also observed as a consequence of matings with the inositol negative *E. coli* Hfr strain.

The linkage relationships of the biochemical characteristics exhibited by the *S. typhimurium* hybrids as determined in these preliminary experiments seem to be the following: Lac-Ara-Rha-Inos-Ind. Also indicated is the finding that 1 per cent of the hybrids selected for rhamnose utilization were both lactose and indol positive, although unchanged for the utilization of inositol. In contrast, no instance of a lactose and indol positive hybrid has been encountered when lactose was employed as the selective marker.

A number of characters present in the *E. coli* parent strain could not be detected in the recombinants when examined as either selected or unselected markers. For example, a *d*-arabinose positive mutant of the *E. coli* Hfr strain was mated with strain TM-9S^r-2. Selection on minimal *d*-arabinose agar or EMB *d*-arabinose agar failed to yield recombinants, while recombinants selected at the Lac locus remained *d*-arabinose negative. A cystine requiring mutant of TM-9S^r-2 obtained by the penicillin method described by Lederberg[23] likewise did not yield recombinants on minimal glucose agar.

Response of hybrids to bacteriophages: The T series of coliphages was tested for lytic ability on *S. typhimurium* strain TM-9S^r-2 with results that indicated host adaptation. When 10^9 particles of T_2 or T_6 (as titered on *E. coli* B) were plated on TM-9S^r-2, not more than 2 to 3 plaques were observed. The phage from these plaques could be propagated on TM-9S^r-2 which were barely visible. The most useful result from the standpoint of genetic study was obtained with a phage originally characterized as a Salmonella phage when isolated at the Army Medical Service Graduate School in 1936. This phage, which will be referred to as phage R, was examined and found to be capable of lysing rough and smooth strains of many species of Shigella, Salmonella, as well as a number of strains of *E. coli*. R phage plated on *E. coli* B, K-12, and *S. typhimurium* produced confluent lysis on these strains. Resistant colonies were observed, although in the case of *E. coli* K-12, these were predominantly mucoid. Further study of resistance to these viruses as genetic markers is planned.

The behavior exhibited by the hybrids toward phage PLT-22 isolated by Zinder and Lederberg[12] was also studied. The parent strain of *S. typhimurium* was sensitive to this phage and showed similar titers to those obtained with *S. typhimurium* LT-2 as indicator strain. It was found that all hybrids obtained following selection for lactose were equally sensitive to PLT-22. When recombinants were selected for *l*-arabinose utilization, approximately 25 per cent of the hybrids obtained were sensitive, an equal number were now resistant to plaque formation by this phage, while the remainder of the hybrids were sensitive, but with low efficiency of plating. Though all of the hybrids tested were identical in antigenic makeup and similar in colonial appearance it is still possible that the differences in sensitivity to PLT-22 may have been due to undetected antigenic changes which may be independent of the mating process. Examination of the parent culture by means

of a dissecting microscope with oblique lighting has revealed some differences in colonial morphology. The significance of these results has not yet been determined.

Although PLT-22 is unable to transmit genetic characteristics directly from *E. coli* to Salmonella, since its host range is limited to Salmonella species possessing the XII antigen and it cannot be grown on *E. coli;* it, nevertheless, could be useful in the transduction of genetic material from *E. coli* × Salmonella hybrids to other susceptible strains of Salmonella. For this reason, lysates of PLT-22 were prepared using a sensitive lactose positive hybrid of *S. typhimurium* TM-98^r-2. Initial attempts to introduce the lactose marker into other species of Salmonella using preparations containing 10^{10} particles/ml of PLT-22 were unsuccessful, although in these same experiments, this preparation was competent, for example, in the transduction of arabinose utilization to strains of *S. typhosa*. Further attempts with higher-titered phage preparations of PLT-22 (5×10^{11} particles/ml) have resulted in the isolation of Lac$^+$ *S. typhosa* strains at a low frequency; Vi phage lysates of a Lac$^+$ *S. typhosa* strain (also Ara$^+$, Xyl$^+$, Rha$^+$) were prepared in the manner previously described by Baron *et al.*,[16] and used to transduce these factors into a wild type strain of *S. typhosa* (Lac$^-$ Ara$^-$ Xyl$^-$ Rha$^-$). Again transduction to lactose utilization was a rare occurrence, while arabinose positives appeared at high frequency; the frequency of transduction to xylose or rhamnose utilization occurred at a lower rate than that observed for arabinose or nutritional markers using either Vi phage or PLT-22, a result which had been reported earlier.[24, 25] These systems may prove useful in genetic analysis of linkage relationships of *S. typhosa* for confirmatory purposes, since it has been possible to mate *S. typhosa* with *E. coli* at a high frequency.[26]

Transfer of lysogenicity for phage lambda[27] to any of a large number of recombinants of *S. typhimurium* from the lysogenic *E. coli* donor strains has not been detected in these experiments. It should be noted, however, that the strains of Salmonella which were studied were not sensitive to lambda phage nor were they susceptible to lysogenization by this phage under usual conditions.

Serology of the hybrids: At present, all of the hybrids isolated at the various selective loci have not been subjected to extensive antigenic analyses for possible changes in somatic or flagellar antigens. Minor cross reactions have been noticed with some hybrid strains of *S. typhimurium* when tested with anti-K-12 serum; all the Lac$^+$ hybrids of *S. typhimurium* which were examined, however, retained their *known* somatic and flagellar components. These hybrids were studied as completely as possible, since Luria and Burrous[21] have reported that a majority of Lac$^+$ hybrids obtained from crosses of *E. coli* and *Shigella flexneri* strain 2aI show a markedly altered serological behavior. In addition, the Lac$^+$ Salmonella hybrids were tested with antisera to Shigella hybrid F22, with this strain as a control, to determine whether such a change had occurred in the *S. typhimurium* hybrids. None of the Lac$^+$ *S. typhimurium* hybrids tested showed any cross-reactivity with anti-F22 serum.

Polarity of the recombinants: A number of attempts have been made to determine the sexual polarity of the *S. typhimurium* hybrids. Lactose selected recombinants obtained from a mating with an F$^+$ *E. coli* strain did not mate with either F$^-$ *E. coli*, or with other Salmonella.

Discussion.—The experiments which are reported here have shown that at least

some species of Salmonella are able to mate with *E. coli*. In addition, the results may provide a further tool for the study of the genetic behavior of *S. typhimurium*, a problem which has been studied primarily by means of transductional recombination as reviewed by Hartman.[28]

The chance isolation, prior to selective recombination, of a high frequency recipient strain supports the possibility that many species of Salmonella may possess cells exhibiting the attributes of F^- or recipient organisms. It is also likely that the rare hybrids which can be obtained from crosses of other strains of Salmonella with *E. coli*, would now act as high frequency recipient organisms on remating with *E. coli*. There are a number of interpretations for the lower frequency of recombination which is evident even in the case of the TM-9S^r-2 strain of *S. typhimurium* when compared with results which have been obtained in *E. coli* Hfr × *E. coli* F^- crosses. Thus, it is conceivable that the TM-9S^r-2 strain represents a form capable of becoming F^- at a higher frequency than strain TM-9 from which it was derived. It may, therefore, not be the typical or ultimate F^- strain of Salmonella which might occur. Another interpretation bears on the possibility of a relatively infrequent integration of the genetic material transferred from the *E. coli* parent to the Salmonella recipient. It would be premature, however, to make any statements concerning the degree of genetic homology between *E. coli* and *S. typhimurium* even though results have indicated that certain of the characteristics of the *E. coli* parent could not be detected in the hybrids. The extent of genetic homology between these organisms may be approached more significantly through the use of different Hfr strains of *E. coli* as has been described by Wollman *et al.*[29]

There may be a number of problems which can be examined effectively by further application of this means of bacterial hybridization. Thus, the problem of the virulence for mice of *S. typhimurium*, investigated by many workers throughout the years, may now be amenable to genetic analysis. Along these lines, preliminary experiments have been initiated to determine if any hybrids of *S. typhimurium* have lost virulence as a consequence of recombination with the mouse avirulent parent strain of *E. coli*. The correlation of antigenic or other types of changes associated with loss of virulence could prove useful for the possible determination of a genetic basis for virulence.

Although preliminary experiments using Salmonella as donor organisms (either F^+ or Hfr) have not been successful, it would still seem feasible for such strains to be isolated within the existing compatibility framework. It should be possible under certain circumstances, with appropriate mating strains and proper selective procedures to transfer Hfr ability to Salmonella species. As a result, it would not be unreasonable to expect successful crosses of Salmonella × Escherichia, Salmonella × Salmonella, or Salmonella × Shigella. The hybrids of such crosses would surely offer material of an exceptionally interesting nature for studies in bacterial virulence.

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*ROLE OF EPISTASIS AND OVERDOMINANCE IN STABILITY OF EQUILIBRIA WITH SELECTION**

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The condition which leads to a stable equilibrium under constant selective values for a single locus with two alleles is simply heterozygote superiority. This condition is also necessary and sufficient for many loci as long as they recombine freely and do not exhibit epistasis. The purpose of this study is to investigate the conditions of stable equilibria when genes do exhibit epistasis. Two alleles per locus, constant genotypic values, free recombination, and random mating are assumed throughout.

Genotypic Variance as Functions of Derivatives of the Mean.—The conditions for stable equilibria must be phrased in terms of derivatives of the mean of a population. These derivatives are essentially gene effects (mono-, di-, tri-genic, and so on) which lead to the same partitions of genotypic variance as those of Cockerham[1] and Kempthorne.[4] This method of obtaining the partitions will be briefly illustrated to establish notation and to tie the conditions for stable equilibria as closely as possible to the genotypic variances. It is worth noting also that the procedure is expeditious

in obtaining the partitions of genotypic variance for many genetic models where the mean can be written as a condensed function of gene frequencies and parameters of the model.

The mean of the population, $\bar{Y}$, may be written as

$$\bar{Y} = q_k^2 Y_{2k} + 2q_k(1 - q_k)\bar{Y}_{1k} + (1 - q_k)^2 \bar{Y}_{0k}, \tag{1}$$

where q_k is the frequency of allele, A, at the kth locus, and $\bar{Y}_{2k}$, $\bar{Y}_{1k}$ and $\bar{Y}_{0k}$ are the mean of three groups of individuals according to the three genotypes A_kA_k, A_ka_k and a_ka_k, respectively. The partial derivative of the mean (1) with respect to q_k is

$$\frac{\delta \bar{Y}}{\delta q_k} = 2(a10)_k = 2\{q_k(\bar{Y}_{2k} - \bar{Y}_{1k}) + (1 - q_k)(\bar{Y}_{1k} - \bar{Y}_{0k})\}, \tag{2}$$

Expression (2) was used by Wright[8] to formulate the change in gene frequencies due to differential selective values of genotypes. It is twice the average excess or average effect (they are the same in random mating populations) of the kth locus defined by Fisher.[2, 3] The contribution of the kth locus to the additive genetic variance, σ_{10}^2, is

$$(\sigma_{10}^2)_k = 2q_k(1 - q_k)(a10)_k^2 \text{ and } \sigma_{10}^2 = \sum_{k=1}^{s} (\sigma_{10}^2)_k, \tag{3}$$

where s is the total number of loci.

The second derivative of the mean with respect to q_k is

$$\frac{\delta^2 \bar{Y}}{\delta q_k^2} = 2(a01)_k = 2\{\bar{Y}_{2k} - 2\bar{Y}_{1k} + \bar{Y}_{0k}\}, \tag{4}$$

which is twice the dominance comparison for the kth locus. The contribution of the kth locus to the dominance variance, σ_{01}^2, is

$$(\sigma_{01}^2)_k = q_k^2(1 - q_k)^2 (a01)_k^2 \text{ and } \sigma_{01}^2 = \sum_{k=1}^{s} (\sigma_{01}^2)_k, \tag{5}$$

The mixed partial derivative of the mean with respect to q_k and q_j is

$$\frac{\delta^2 \bar{Y}}{\delta q_k \delta q_j} = 4(a20)_{jk} = 4\{q_k q_j l_{2k2j} + q_k(1 - q_j) l_{2k1j} + (1 - q_k)\, q_j l_{1k2j} + (1 - q_k)(1 - q_j)\, l_{1k1j}\}, \tag{6}$$

where

$$l_{2k2j} = \bar{Y}_{2k2j} - \bar{Y}_{2k1j} - \bar{Y}_{1k2j} + \bar{Y}_{1k1j},\ l_{2k1j} = \bar{Y}_{2k1j} - \bar{Y}_{2k0j} - \bar{Y}_{1k1j} + \bar{Y}_{1k0j}$$
$$l_{1j2k} = \bar{Y}_{1k2j} - \bar{Y}_{1k1j} - \bar{Y}_{0k2j} + \bar{Y}_{0k1j},\ l_{1k1j} = \bar{Y}_{1k1j} - \bar{Y}_{1k0j} - \bar{Y}_{0k1j} + \bar{Y}_{0k0j}$$

and $\bar{Y}_{2k2j}$, $\bar{Y}_{2k1j}$, . . ., $\bar{Y}_{0k0j}$ are the means of individuals with genotypes, $A_kA_kA_jA_j$, $A_kA_kA_ja_j$, . . ., $a_ka_ka_ja_j$, respectively. This expression (6) is the additive × additive comparison for the kth and jth loci. The contribution of the kth and jth loci to the additive × additive variance, σ_{2c}^2, is

$$(\sigma_{20}^2)_{kj} = 4q_k(1 - q_k)q_j\,(1 - q_j)(a20)_{kj}^2 \text{ and } \sigma_{20}^2 = \sum_{k<j=1}^{s} (\sigma_{20}^2)_{kj}, \tag{7}$$

The expressions (3), (5), and (7), and other partitions of variance are the same as those given by Cockerham,[1] when there is no inbreeding.

$$\text{Generally, if a quantity, } (aLQ) = \frac{1}{2^{(L+Q)}} \frac{\delta^{(L+2Q)}\bar{Y}}{\prod_j^L \delta q_j \prod_k^Q \delta q_k^2} \text{ for } j \neq k, \tag{8}$$

then a contribution of the comparison (L-additive) $\times$ (Q-dominance) from a set of ($L + Q$) loci to the (L-additive) $\times$ (Q-dominance) variance is

$$(\sigma_{LQ}^2) = 2^L \prod_j^L q_j(1 - q_j) \prod_k^Q q_k^2 (1 - q_k)^2 (aLQ)^2, \tag{9}$$

Conditions for Stable Equilibria.—Consider a surface of the means of a given character with respect to s coordinates, each corresponding to the gene frequency of one of s loci. When the character is a measure of the adaptive value of genotypes, such a surface is called the adaptive surface.[7] The method of finding the conditions for an equilibrium to be stable under mass selection is to investigate the conditions for a point on the surface to be a relative maximum, i.e., a slight shift of gene frequencies in any direction results in a lower mean.

For a given point ($\bar{Y}$, q_1, q_2, . . ., q_s) on the surface to be a relative maximum, the following conditions are necessary and sufficient:

i.

$$\frac{\delta \bar{Y}}{\delta q_j} = 0 \qquad \text{for } j = 1, 2, \ldots, s. \tag{10}$$

Let the nontrivial solutions (i.e., $q_j \neq 0$ or 1) of the simultaneous equations in (10) be ($\hat{q}_1$, $\hat{q}_2$, . . ., $\hat{q}_s$).

ii. The quadratic form, of which the element d_{jk} is $\delta^2 \bar{Y}/\delta q_j \delta q_k$ for all j and k ($j = 1, 2, \ldots, s$; $k = 1, 2, \ldots, s$), must be negative definite at the point ($\hat{q}_1$, $\hat{q}_2$, . . ., $\hat{q}_s$). This means that the principal minors, J_l, of the matrix of the quadratic form must have sign $(-1)^l$ for $l = 1, 2, \ldots, s$.

The genetic implication of these two conditions are now considered. From (2) the solutions of the equations in (10) have the form

$$\hat{q}_j = \frac{1}{1 + \dfrac{\bar{Y}_{1j} - \bar{Y}_{2j}}{\bar{Y}_{1j} - \bar{Y}_{0j}}} \qquad \text{for } j = 1, 2, \ldots, s. \tag{11}$$

This indicates that two differences ($\bar{Y}_{1j} - \bar{Y}_{2j}$) and ($\bar{Y}_{1j} - \bar{Y}_{0j}$) must have the same sign for each j in order that the $\hat{q}$'s be nontrivial. In other words the heterozygote must be either superior or inferior to both homozygotes in terms of marginal means of three genotypes at each locus. This is a necessary condition for a point to be an equilibrium.

From (4) and (6), the elements d_{jk} in the quadratic form are identified as

$$\begin{aligned} d_{jj} &= 2(a01)_j \\ d_{jk} &= 4(a20)_{jk} \end{aligned} \tag{12}$$

where d_{jj}'s are diagonal elements and d_{jk}'s are off-diagonals. Then the sufficient conditions for a point of equilibrium $(\hat{q}_1, \hat{q}_2, \ldots, \hat{q}_s)$ to be stable is

$$\begin{array}{lll} (a01)_j & < 0 \text{ for all } j & c_1 \\ \begin{vmatrix} (a01)_j & 2(a20)_{jk} \\ 2(a20)_{jk} & (a01)_k \end{vmatrix} & > 0 \text{ for all } j \text{ and } k & c_2 \\ \begin{vmatrix} (a01)_j & 2(a20)_{jk} & 2(a20)_{jm} \\ 2(a20)_{jk} & (a01)_k & 2(a20)_{km} \\ 2(a20)_{jm} & 2(a20)_{km} & (a01)_m \end{vmatrix} & < 0 \text{ for all } j, k \text{ and } m & c_3 \\ \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot \\ \cdot & \cdot & \cdot \end{array} \tag{13}$$

Since the dominance comparisons are of the form $(\bar{Y}_{2j} - 2\bar{Y}_{1j} + \bar{Y}_{0j})$ and must be negative, the equilibrium is unstable when the heterozygote is inferior to the both homozygotes just as it is when the loci do not interact.

These conditions, $c_1, c_2, \ldots, c_3$ may be viewed stepwise. For a single locus or noninteracting loci only heterozygote superiority of each is sufficient. For two loci which interact an additional condition, c_2, is necessary for them to be jointly held in a stable equilibrium. For three interacting loci, not only do c_1 and c_2 have to hold but also c_3 must be satisfied for them to be simultaneously kept in a stable equilibrium and so on for more loci. These conditions are not easy to visualize without making some simplification. The general case for two loci will be considered, and for more loci, they will be considered to be identical in their effects.

For $s = 2$ the inequality c_2 in (13) limit the magnitude of the additive × additive comparison in such a way that

$$(a01)_1 \cdot (a01)_2 > 4(a20)_{12}^2, \tag{14}$$

From (9) the inequality (14) is equivalent to saying that the geometric mean of the two dominance variances must be larger than the additive × additive variance for the two loci, i.e., $(\sigma_{01})_1 \cdot (\sigma_{01})_2 > (\sigma_{20}^2)_{12}$ which is $\sigma_{01}^2 > 2\sigma_{20}^2$ if $(\sigma_{01})_1 = (\sigma_{01})_2$.

For more than two loci the simplification that all loci have equal effects and that they have the same gene frequencies when at equilibrium, will be imposed. Then the dominance comparisons are the same, and the additive × additive comparisons are the same for all loci. The conditions now simplify to

$$\begin{array}{ll} (a01) < 0 & c_1 \\ 0 < (a20) < -(a01)/2(s-1), \text{ if } (a20) > 0 & c_2' \end{array}$$

or

$$(a01) < 2(a20) < 0, \text{ if } (a20) < 0 \qquad c_2''$$

The first condition, c_1, is the same one insuring heterozygote superiority, and in addition, one of two conditions, c_2' or c_2'', which limits the additive × additive comparison relative to the dominance comparison, is necessary for the s loci to be jointly held in a stable equilibrium.

Translating c_2' and c_2'' into variances, these conditions are

$$\sigma_{20}^2 < \sigma_{01}^2/2(s-1), \text{ if } (a20) > 0 \qquad c_2'$$
$$\sigma_{20}^2 < \frac{(s-1)}{2}\sigma_{01}^2, \quad \text{if } (a20) < 0 \qquad c_2''$$

Thus, if the additive × additive comparison is positive, the amount of additive × additive variance relative to the dominance variance is severely limited for an equilibrium to be maintained, and when there are many loci. On the other hand if $(a20)$ is negative, σ_{20}^2 can be as large as $(s-1)/2$ times σ_{10}^2 for an equilibrium to be stable. The two conditions are the same when only two loci are considered.

Discussion.—The results of this study are specifically for two alleles at each locus. The conditions for stable equilibria of interacting loci with multiple alleles will probably be very different from those for interacting loci with only two alleles. Kimura[5] gave algebraic rules for testing stability of equilibria for multiple alleles at a single locus and indicated that simple heterozygote superiority was no longer a necessary condition for stable equilibrium.

The assumption of constancy of genotypic values may be valid in certain situations but not in others. It is not clear how large the fluctuations in genotypic values can be, random or otherwise, and the conditions derived in this paper still be valid. In some cases the genotypic values may vary as functions of gene frequencies as was considered by Wright[9] and Lewontin[6] for a single locus with two alleles. Lewontin found that even heterozygote superiority was not necessarily required for a stable equilibrium.

Although only unlinked genes are considered, linkage disequilibrium can arise from selection even when random mating is maintained. This linkage disequilibrium is ignored in this paper. However, the amount of linkage disequilibrium depends largely on the amount of shift in genotypic frequencies each generation. Since changes in genotypic frequencies are small near points of equilibria, it is believed that the effect of linkage disequilibrium is trivial and that the conditions found for stable equilibria are valid. The effect of linkage and linkage disequilibrium needs further study.

While general conditions for stable equilibria of two loci were given in terms of components of variance, this could not be done for more than two loci without making restrictions. The restrictions of equal effects and equal frequencies of genes are unrealistic. However, the results indicate that generally only so much additive by additive epistatic variance can be afforded relative to the dominance variance and an equilibrium be maintained.

Summary.—The conditions for maintaining stable equilibria in a population undergoing mass selection and for interacting genes are studied. For two loci to be jointly held in a stable equilibrium, overdominance is required at each locus and the geometric mean of the dominance variances from each locus must be larger than the additive × additive variance for two loci. For more than two loci explicit expressions in terms of partitions of genotypic variance are given for stable equilibria when all loci have equal effects and equal frequencies. Roughly speaking in the general case, more overdominance tends to maintain stable equilibria, while more additive × additive epistasis tends to destroy stable equilibria.

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STABLE EQUILIBRIA FOR THE OPTIMUM MODEL

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The optimum model proposed by Wright[4] has been used to demonstrate theoretical outcomes in a mendelian population when selecting for an intermediate phenotype.[2, 3, 6] Whether or not this model leads to stable equilibria, however, has not been investigated except in a few special cases.[5] The purpose of this paper is twofold: to illustrate the method[1] of finding stable points of equilibria with respect to mass selection when loci exhibit epistasis, and to indicate that such points do exist for the optimum model.

Wright[4] introduced the optimum model,

$$Y = -(S - \Phi)^2$$

where S was the underlying (genotypic) value on the primary scale, Φ was the optimum or most desired value on the primary scale and Y was the observed or phenotypic value on the secondary scale. In a subsequent paper[5] he investigated the quasi-static evolution of a population in which the selective values of individuals were proportional to their values on the secondary scale. The contributions of the loci to the primary scale, S, were always considered to be additive among loci. Two cases of dominance, either no dominance or complete dominance at all loci were considered. In either case stable equilibria were not found.

Actually, other levels of dominance (partial or overdominance) on the primary scale produce stable equilibrium points. To illustrate that this is so, suppose the primary value, S, is determined additively by the loci such that the jth locus contributes α_j, $h_j\alpha_j$, and $-\alpha_j$ corresponding to the phases A_jA_j, A_ja_j, and a_ja_j of the locus. Without loss of generality α can always be considered to be positive. The value of h reflects dominance on the primary scale as follows:

$h = 0$	no dominance
$\|h\| < 1$	partial dominance
$h = \pm 1$	complete dominance
$\|h\| > 1$	overdominance

where $|h|$ is absolute value of h. The mean, $\bar{Y}$, of the secondary values in a random mating population is

$$\bar{Y} = -\{(\bar{S} - \Phi)^2 + V\}$$

where $\bar{S}$ is the mean of primary values and V is the total genotypic variance on the primary scale. Let P_j be the mean contribution of the jth locus to S, then

$$P_j = q_j^2\alpha_j + 2q_j(1 - q)_j\, h_j\alpha_j - (1 - q_j)^2\alpha_j$$

and

$$\bar{S} = \sum_{j=1}^{n} P_j$$

where q_j is the relative frequency of the allele A_j, and n is the number of loci. The variance, V, is equal to

$$\sum_{j=1}^{n} \{q_j^2\alpha_j^2 + 2q_j(1 - q_j)\, h_j^2\alpha_j^2 + (1 - q_j)^2\alpha_j^2 - P_j^2\}$$

when the loci are in linkage equilibrium.

By taking successive derivatives[1] of the mean, $\bar{Y}$, with respect to two gene frequencies q_j and q_k, the following additive, dominance and additive $\times$ additive comparisons are obtained:

additive ($^1/_2$ of first derivative):

$$(a10)_j = -[\alpha_j^2(h_j^2 - 1)\,(1 - 2q_j) + 2\alpha_j\,\{1 + h_j(1 - 2q_j)\}\,(\bar{S} - P_j - \Phi)] \quad \text{for } j = 1, 2, \ldots, n$$

dominance ($^1/_2$ of second derivative):

$$(a01)_j = [2\alpha_j^2(h_j^2 - 1) + 4h_j\alpha_j(\bar{S} - P_j - \Phi)] \text{ for } j = 1, 2, \ldots, n$$

additive $\times$ additive ($^1/_4$ of mixed derivative):

$$(a20)_{jk} = -2\alpha_j\alpha_k\,\{1 + h_j\,(1 - 2q_j)\}\,\{1 + h_k(1 - 2q_k)\} \quad j > k = 1, 2, \ldots, n.$$

These are the quantities needed to determine the points of equilibria and their stability.

The conditions for the points of equilibria to be stable when many genes are interacting have been derived.[1] In order to point out the existence of stable equilibria in the optimum model, only models for two loci will be considered, although stability of equilibria in any other model can be determined by the interrelations of two types of comparisons, $(a01)_j$ and $(a20)_{jk}$. The procedure to find stable equilibria of two loci can be described as follows:

1. Solve $(a10)_1 = 0$ and $(a10)_2 = 0$ simultaneously with respect to the gene frequency of each locus. They are the equilibrium gene frequencies, $\hat{q}_1$ and $\hat{q}_2$, if both are not trivial, i.e., $0 < \hat{q}_1 < 1$ and $0 < \hat{q}_2 < 1$.

2. Compute $(a01)_1$ and $(a01)_2$ at $\hat{q}_1$ and $\hat{q}_2$. Both quantities must be negative for the point of equilibrium to be stable. When $(a01)_1 < 0$ and $(a01)_2 < 0$ at non-trivial values of $\hat{q}_1$ and $\hat{q}_2$, the marginal means for heterozygotes of both loci are larger than the marginal means for the corresponding two homozygotes at each locus. That is to say, the heterozygotes are superior to the homozygotes at both loci.

3. Finally compute the value of $(a20)_{12}$ at $\hat{q}_1$ and $\hat{q}_2$. For the equilibrium to be stable the inequality,

$$(a01)_1 \cdot (a01)_2 > 4(a20)_{12}^2$$

must hold. When such an inequality is true, the geometric mean of the two dominance variances must be larger than the additive × additive variance when at $\hat{q}_1$ and $\hat{q}_2$[1].

Usually a trial and error procedure is needed to solve for $\hat{q}$'s in the equations of the $a(10) = 0$ type. Some numerical examples for two loci with the same values of α and h are given in the following.

Example 1: $\alpha = 2$, $h = 0.5$ and $\Phi = 2.5$. An equilibrium is found at $\hat{q}_1 \doteqdot 0.86$ and $\hat{q}_2 \doteqdot 0.86$. $(a01)$'s are -9.28, then heterozygote superiority on the marginal means holds at this point, and

$$(a01)_1 \cdot (a01)_2 = 86.12 \text{ while } 4(a20)_{12}^2 = 43.00.$$

Then this is a stable equilibrium.

Example 2: $\alpha = 2$, $h = 0.5$ and $\Phi = 2.0$. An equilibrium point locates at $\hat{q}_1 \doteqdot 0.77$ and $\hat{q}_2 \doteqdot 0.77$. Both dominance comparisons are -8.26, then heterozygote superiority holds at this point, but

$$(a01)_1 \cdot (a01)_2 = 68.28 \text{ and } 4(a20)_{12}^2 = 72.70$$

Hence this point is an unstable equilibrium.

Example 3: $\alpha = 6$, $h = 0.9$ and $\Phi = 6$. Symmetric equilibria are found at $\hat{q}_1 \doteqdot 0.95$ and $\hat{q}_2 = 0.57$ and at $\hat{q}_1 = 0.57$ and $\hat{q}_2 = 0.95$. $(a01)_1 = -13.87$ and $(a01)_2 = -60.18$, then heterozygote superiority holds at both points. When at $q_1 = 0.95$ and $q_2 = 0.57$, $(a01)_1 \cdot (a01)_2 = 834.70$ which is larger than $4(a20)_{12}^2 = 602.84$. Since the model is symmetric, this relation is also true for the other point. Hence these two points are stable equilibria.

The conditions for marginal overdominance can be more fundamentally expressed in terms of Φ, α, h and q. Let $\bar{Y}_{2j}$, $\bar{Y}_{1j}$ and $\bar{Y}_{0j}$ be the marginal means of the three genotypes, A_jA_j, A_ja_j and a_ja_j, respectively. Then the conditions are

$$\bar{Y}_{2j} - \bar{Y}_{1j} = (h_j - 1)\alpha_j \{2(\bar{S} - P_j - \Phi) + \alpha_j(h_j + 1)\} < 0$$

and

$$\bar{Y}_{0j} - \bar{Y}_{1j} = (h_j + 1)\alpha_j \{2(\bar{S} - P_j - \Phi) + \alpha_j(h_j - 1)\} < 0$$

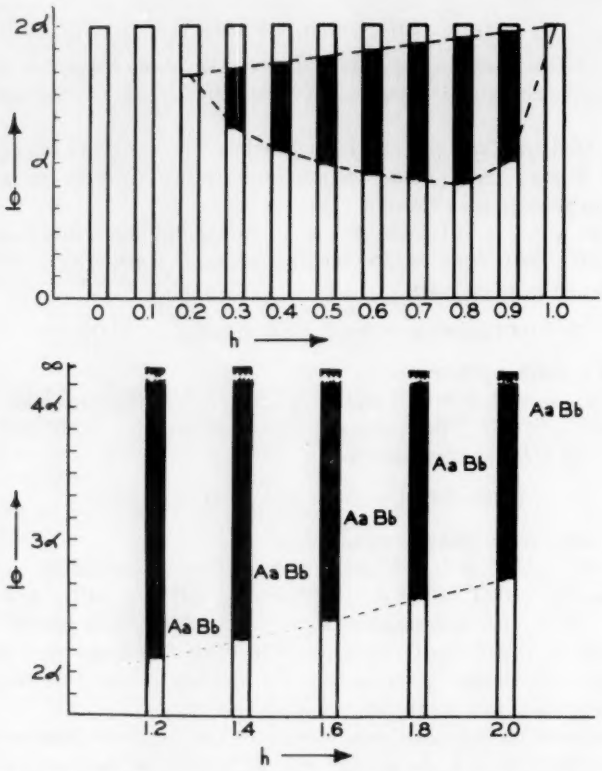
which restrict Φ to the following ranges,

$$\bar{S} - P_j + \frac{\alpha_j(h_j - 1)}{2} < \Phi < \bar{S} - P_j + \frac{\alpha_j(h_j + 1)}{2} \text{ when } |h_j| < 1$$

$$\bar{S} - P_j + \frac{\alpha_j(h_j + 1)}{2} < \Phi \text{ when } h_j > 1, \text{ and}$$

$$S - P_j + \frac{\alpha_j(h_j - 1)}{2} < \Phi \text{ when } h_j < -1.$$

It is important to note that stable equilibria can exist in the optimum model with an intermediate optimum value between two extreme values on the primary scale. In Figures 1 and 2 the magnitudes of Φ and h which produce stable equilibria for two loci are shown for $0 \leq h \leq 1$ and $h > 1$, respectively. The two loci are assumed



Figs. 1 and 2.—Values of the optimum which give rise to stable equilibria for various levels of dominance in the primary scale. Two loci with the same effects are assumed. Figure 1 is for partial dominance and Figure 2 is for overdominance on the primary scale.

to have the same α and same h. The shaded regions represent the conditions resulting in stable equilibria with nontrivial gene frequency.

For $0 \leq h \leq 0.2$, there is no value of the optimum which results in stable equilibria. When h increases the shaded region increases, but as h approaches 1 the shaded region disappears rapidly. As the value of h continues to increase into the overdominance range on the primary scale (Fig. 2), the shaded region picks up again. Although the value of h is given only up to 2.0 in Figure 2, the results can be easily visualized for $h > 2$.

Summary.—The existence of stable equilibria in Wright's optimum model has been demonstrated. They required either partial or overdominance on the primary scale of gene effects. Some examples were given for two loci.

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PLEIOTROPY AND COMPETITION AT THE VERMILION LOCUS IN DROSOPHILA MELANOGASTER

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Recessive vermilion (v) mutants in *Drosophila melanogaster*, although phenotypically indistinguishable, may be separated into two classes in the presence of a linked suppressor mutant.[1] One class of mutants (designated collectively as v^s) is modified to wild type in the presence of the suppressor, and the other class (designated collectively as v^n) is unaltered.[1] In v mutants, a failure occurs in the conversion of tryptophan to kynurenine, this resulting in a block in the synthesis of brown eye pigment. When v larvae are fed kynurenine, or formylkynurenine, which is the immediate precursor of kynurenine, brown eye pigment is synthesized.[1] The biochemical block therefore occurs somewhere between trytophan and formylkynurenine.

The possibility exists that v flies able to synthesize brown pigment due to the presence of the suppressor gene are fitter than those unable to synthesize it. If the suppressor is ineffective, as in v^n mutants, then the fitness of v flies with and without the suppressor should be indistinguishable, but when the suppressor is effective, as in v^s mutants, the fitness of v flies with the suppressor should be greater than unsuppressed flies. This permits a test of the possibility that eye color synthesis and fitness are related to the same biochemical reaction.

In the experiments to follow, competitiveness under very crowded conditions will be used as a criterion of fitness. This has the advantage of giving a possible conclusion in one generation rather than several generations as in a population cage experiment. Under conditions of low competition little effect should be observed, but under high competition, if there is a difference in competitiveness between competing genotypes, then it may be detected.[2, 3]

Competition Experiments.—Stocks of v^1, $su^2{-}s\ v^1$, v^{36f}, $su^2{-}s\ v^{36f}$ ($su^2{-}s$ = suppressor of v) in a homozygous brown (*bw*) background were used. In the presence of *bw*, v^1, v^{36f} and $su^2{-}s\ v^{36f}$ flies have white eyes and $su^2{-}s\ v^1$ brown eyes. To be

strictly accurate, v^1; *bw* flies have eyes which are very faintly brown. The brown color is intensified under conditions of larval starvation,[4] or as the flies become older. Thus v^1 is a v^s mutant and v^{36f} a v^u mutant.

The females of these four stocks were crossed to homozygous *bw* males under conditions of low and high competition and the sex ratio noted. In all cases the female progeny appeared brown as they are heterozygous for the *v* gene. However, the males are v^1; *bw*, $su^2-s\ v^1$; *bw*, v^{36f}; *bw* and $su^2-s\ v^{36f}$; *bw* according to the cross, and appear white with the exception of the brown $su^2-s\ v^1$; *bw* males. Essentially, therefore, v^+ females are competing against *v* males.

One hundred eggs of the relevant mating in a small vial with 9 gm of food made up a replicate for the low level of competition, and 500 eggs in a small vial with 3 gm of food for the high level. At the low level, flies were counted daily until they had all emerged. At the high level, flies were counted daily up to the tenth day from the day when the flies commenced to emerge. This was shown to be equivalent to classifying the culture completely as very few flies emerged after ten days.

At the low level of competition, there was no significant difference between flies with and without the suppressor (Table 1). Replicates within each mating were

TABLE 1

SEX RATIO FOR LOW AND HIGH COMPETITION FOR THE CROSSES INVOLVING v^1, v^{36f}, AND $su^2 - s$

Cross ♀ × ♂	Females, (%)	Males	Total	Contingency χ^2
		Low Competition		
v^1; *bw* × *bw*	346 (55.9)	273	619	0.34 ($0.5 < P < 0.7$)
$su^2 - s\ v^1$; *bw* × *bw*	408 (54.2)	345	753	
v^{36f}; *bw* × *bw*	397 (51.7)	371	768	1.10 ($0.2 < P < 0.3$)
$su^2 - s\ v^{36f}$; *bw* × *bw*	354 (53.8)	294	658	
		High Competition		
v^1; *bw* × *bw*	605 (60.0)	403	1,007	6.61 ($P < 0.02$)
$su^2 - s\ v^1$; *bw* × *bw*	601 (54.3)	505	1,106	
v^{36f}; *bw* × *bw*	677 (54.5)	565	1,242	0.001 ($P > 0.9$)
$su^2 - s\ v^{36f}$; *bw* × *bw*	725 (54.5)	605	1,330	

not heterogeneous. The low level will therefore act as a control for the high level. Usually, 90 to 100 flies emerged for each replicate for all crosses indicating that the hatchability in each cross was roughly equivalent. Hence the level of competition at the high level is equivalent for each cross.

At the high level, there was no significant difference between the matings v^{36f}; *bw* × *bw* and $su^2-s\ v^{36f}$; *bw* × *bw*. In the $su^2-s\ v^{36f}$; *bw* stock the suppressor has no detectable effect which is in agreement with expectation. The sex difference in the mating $su^2-s\ v^1$; *bw* × *bw* was smaller than for v^1; *bw* × *bw*. The suppressor therefore has an effect in the v^1; *bw* stock, and this suggests that the suppressor, when active, results in the increased fitness of the males because of the eye pigment synthesis. This result is only strongly suggestive, for the possibility of a genetic background effect cannot be excluded. However, in the next section, the validity of this conclusion is confirmed.

The Effect of Kynurenine on Competition.—The suppressor, when active, allows kynurenine to be formed, and hence brown pigment. The addition of kynurenine

to stocks in which the suppressor is absent or inactive produces equivalent phenotypic effects. Hence adding kynurenine may be expected, from the results already presented, to result in increased fitness of unsuppressed stocks. To test this hypothesis, the sex ratios in the matings *v*^{1}; *bw* × *bw* and *v*^{36f}; *bw* × *bw* were estimated with and without kynurenine at the high level of competition. A third mating, *bw* × *bw*, was included as a control.

A solution consisting of 0.05 per cent streptomycin, and 0.033 per cent penicillin was made up. In part of this solution, dl-kynurenine sulfate was dissolved so as to make a 1 per cent kynurenine sulfate solution with streptomycin and penicillin. To one series of replicates, 0.2 ml of the antibiotic solution was added, to a second series 0.1 ml of the antibiotic with 0.1 ml of the antibiotic plus kynurenine, and to a third series 0.2 ml of the antibiotic plus kynurenine solution. These additions were made every two days up to a total of 5 additions so that 1 ml of solution was added to each culture. This represents a total of 0, 2.5, and 5.0 mg. l-kynurenine sulfate to each series of replicates respectively. The isomer d-kynurenine is inactive in brown pigment formation. The antibiotics were added to prevent the metabolism of kynurenine by contaminating bacteria. The adult flies were classified daily up to the tenth day from the first day of emergence.

TABLE 2

SEX RATIOS FOR THE CROSSES *v*^{1}; *bw* × *bw*, *v*^{36f}; *bw* × *bw* AND *bw* × *bw* UNDER VARIOUS LEVELS OF KYNURENINE SUPPLEMENT

	Females	Males	Total	Females, %
	v^{1}; *bw* × *bw*			
Antibiotic only	513	371	884	58.03
2.5 mg l-kynurenine sulfate	401	314	715	56.08
5 mg l-kynurenine sulfate	501	435	936	53.53
	v^{36f}; *bw* × *bw*			
Antibiotic only	575	457	1,032	55.72
2.5 mg l-kynurenine sulfate	596	557	1,153	51.69
5 mg l-kynurenine sulfate	658	625	1,283	51.29
	bw × *bw*			
Antibiotic only	380	372	752	50.53
2.5 mg l-kynurenine sulfate	426	354	780	54.62
5 mg l-kynurenine sulfate	371	312	683	54.32

In Table 2, results are given. Within each treatment, for each mating, the segregations were not heterogeneous.

For the mating *v*^{1}; *bw* × *bw*, the percentage of females decreased with increasing amounts of kynurenine. A contingency χ^2_1 on the results for the antibiotic only, and the high level of kynurenine is 3.59 which may be regarded as suggestive of a difference ($0.05<P<0.1$). Considering this χ^2_1 with the decreasing percentage of females with increasing levels of kynurenine, it can be said fairly conclusively that the addition of kynurenine increases the fitness of the males. The males in the culture to which kynurenine was added had brown eyes, which after three or four days from first emergence were indistinguishable from the females.

Similarly, in the mating *v*^{36f}; *bw* × *bw*, the percentage of females decreased with increasing amounts of kynurenine. A contingency χ^2_1 on the results for the antibiotic only and the high level of kynurenine is 4.33 ($P<0.05$) which is significant. These results, when combined with the evidence from the *v*^{1}; *bw* × *bw* cross show conclusively that kynurenine increases the fitness of the males.

In the control mating *bw* × *bw*, there is, as expected, no significant trend.

Examination of the number of females and males emerging for these cultures shows that the mean number of females emerging for a specific mating type varies little with treatment, but the number of males increases when kynurenine is added (Table 3). This shows that the kynurenine acts by increasing the number of males rather than by decreasing the number of females. This agrees with the hypothesis that the kynurenine increases the fitness of the males. In the control mating *bw* × *bw* this effect is, as expected, not apparent.

TABLE 3

MEAN NUMBER OF FEMALES AND MALES FROM A REPLICATE

	Antibiotic Only	2.5 mg l-kynurenine	5 mg l-kynurenine
	v[1]; *bw* × *bw*		
♀	102.6	100.3	100.2
♂	74.2	78.5	87.0
	v[36f]; *bw* × *bw*		
♀	95.8	99.3	94.0
♂	76.2	92.8	89.3
	bw × *bw*		
♀	76.0	85.2	74.2
♂	74.4	70.8	62.4

The addition of kynurenine therefore has the same effect as the suppressor in conferring added fitness on the males in the two matings *v*[1]; *bw* × *bw* and *v*[36f]; *bw* × *bw*. In the control mating *bw* × *bw*, the addition of kynurenine has, as expected, no significant effect on the sex ratio.

Competition was almost completely larval in these experiments for pupal mortality was not observed, and competition between adults was negligible as each replicate was classified daily. The results presented show that there is a relation between larval fitness and the presence of brown pigment in flies which are genotypically vermilion, whether the brown pigment is supplied by the action of a vermilion suppressor or by the addition of kynurenine. This relation occurs even though brown pigment synthesis takes place in the pupa. Fitness is therefore related to the expression of the vermilion gene, for altering the expression of the gene biochemically or genetically alters the fitness of the gene. Hence the vermilion gene, when expressed, has two pleiotropic effects. One is the inhibition of brown pigment formation because of a biochemical block between tryptophan and kynurenine, and the second is a reduction in fitness compared with flies in which the brown pigment is present. It is likely that both effects are associated with the conversion of tryptophan to kynurenine.

Summary.—Competition experiments were done to determine the effect of suppressors of the vermilion gene on fitness. When the gene is suppressed, the fitness of the suppressed flies is increased under conditions of high competition. The effect of the suppressor on fitness can be imitated by adding kynurenine, which, like the suppressor, permits brown pigment formation. Hence the vermilion gene when expressed has an effect on fitness as well as inhibiting brown pigment formation. Both effects are presumably associated with the conversion of tryptophan to kynurenine. The vermilion gene is therefore pleiotropic.

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INCORPORATION OF H^3 CYTIDINE AND H^3 THYMIDINE INTO GIANT CHROMOSOMES OF DROSOPHILA DURING PUFF FORMATION

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Giant chromosomes of the higher Diptera, exploited since 1932 for their constant morphological features that could be used as cytogenetic markers, are also known to undergo morphological changes concomitant with changes in the metabolic activity of the cells in which they lie. The most commonly observed modification is a local increase in diameter, frequently called a "puff," which is not only restricted to a narrow chromosomal region, but, for a particular chromosome region, is restricted with respect to the cell type in which it is found and to the developmental stage at which it occurs.[1, 2] Elucidation of the chemistry of these localized swellings is one of the most promising approaches to an understanding of the metabolic activities of chromosomes.

At the time of this writing only three quantitative studies have appeared. In the first one, reported in abstract,[3, 4] the ultraviolet absorbing material in a region of a *Drosophila melanogaster* salivary gland chromosome was found to increase slightly but not significantly with respect to neighboring, nonpuff, regions during the course of swelling. On the other hand, the two later studies demonstrated a marked disporportionate synthesis of deoxyribosenucleic acid (DNA) during the swelling of specific regions in salivary gland chromosomes of *Rhynchosciara*[5, 6] and of *Glyptotendipes*.[7] Since no attempt was made to differentiate between the two types of nucleic acid in the *Drosophila* puff the possibility remained that synthesis of DNA, perhaps accompanied by a change in the ratio of DNA to ribosenucleic acid (RNA), had taken place in the puff region. On the other hand, more recent work suggests that the morphological change may be accompanied by a change in RNA: some puffs in *Drosophila virilis*[8] and in other dipteran larvae[9, 10] show staining reactions typical of RNA, and radiophosphorus is incorporated into the RNA of chromosomes of *Drosophila* larvae.[13–15] In order to compare the metabolic activities of puff and nonpuff regions with respect to both RNA and DNA in *Drosophila*, an attempt was made to incorporate tritium labeled thymidine and cytidine into puff regions. The present note is a preliminary report of the results.

Thymidine is known to be specifically incorporated into DNA with no detectable shunting to RNA[16, 17] and, once incorporated, it remains in the DNA with little or no turnover. Thus, an audioradiograph obtained after the administration of label in the pyrimidine nucleus of thymidine is very good evidence that DNA synthesis has taken place at the site of the label. Cytidine is a ribose nucleoside and may be directly incorporated into RNA. However, there is evidence from other organisms[17] that it can also serve as a precursor of pyrimidines in the synthesis of DNA. A distinction may be made in favorable material (such as ours) by comparing the sites of incorporation of each of the two labeled compounds or by the widely used technique of comparing with untreated controls the autoradiographs of specimens from which one or the other nucleic acid has been extracted.[18]

The labeled precursors[19] were injected into larvae (see 11 and 12) by the use of an apparatus patterned after a design by H. K. Mitchell.[20] The amount injected per larva was of the order of 0.05 to 0.1 microliters, corresponding to 0.01 to 0.02 microcuries of tritium in thymidine or to 0.025 to 0.05 microcuries of tritium in cytidine. Normal sized larvae from a stock homozygous for the mutant genes *giant* and *apricot*, but otherwise isogenic with our inbred Oregon R wild type strain, were injected one to seven hours before puparium formation[21] and dissected one-half to four hours after puparium formation. The larvae were raised on corn meal-molasses medium (seeded with live yeast) at 25°C. Dissection and fixation (45 per cent acetic acid) of the salivary glands were carried out on a microscope stage kept near 0°C with cracked ice. The glands were squashed onto microscope slides "subbed" with gelatin. The coverslips were snapped off after the slides had been frozen on dry ice; then the preparations were stored in absolute ethanol overnight in the refrigerator. Stripping film[22] was applied, exposed, and developed in the usual manner.[23] After development of the autoradiographic image some slides were stained with basic fuchsin by the method of Bergeron.[24]

Silver grains were counted over the same chromosome segment in the right end of the second chromosome (2R) that had been previously studied in the ultraviolet, i.e., section 60, as well as the two neighboring sections, numbers 58 and 59 on Bridges' map[25] (see Fig. 4, *a*).

Three types of chromosome region are included in the analysis. The first is the one just mentioned in which a puff appears at the time the puparium is being formed, represented by the proximal portion of section 60 (60AB). The second type is represented by the distal portion of section 58 (58E-F) in which a puff present in the larval chromosome at the time of injection of the labeled precursor persists into the prepupal stage. Finally, the third type in which no puffs are found during the experimental period is represented by the remainder of the chromosome segment (sections 58A-D, 59A-F, and 60C-F). Incorporation of isotope into only one of them could be expected to produce an autoradiographic image of that region alone.

The autoradiographs were inspected under an interference microscope (100× immersion objective, 10× eyepieces) to locate chromosomes 2R that were free of overlying cellular material. The segments to be studied were carefully outlined by making camera lucida drawings of the chromosomes, the areas were measured with a planimeter, and the number of silver grains over each section was counted. Background counts (fog) ranged from 0.04 to 1.0 grain per hundred square micra and have been subtracted from the observed values.

The results with H^3-thymidine were striking. Ten days exposure to the autoradiographic film revealed five per cent of the salivary gland nuclei so heavily labeled that the individual chromosomes could not be identified under the dense layer of silver grains (Fig. 1). Four per cent of the nuclei were lightly labeled and their chromosomes were identifiable (Figs. 2, 3), and the remaining 91 per cent (of 121 nuclei from one salivary gland) were not obviously labeled (Figs. 1, 2). Exposure for thirty days showed only a few more nuclei (12 per cent of 277 nuclei from two glands) in the lightly labeled class, 5 per cent as before in the heavily labeled class, and the remaining 82 per cent not obviously labeled. The same pattern has been observed in eight other animals. The average grain density over 21 unlabeled chromosomes was not significantly above background and the grains

were distributed among all four segments (Table 1). Since most of the chromosomes did not have any grains (above fog) although they exhibited the puffs

TABLE 1

Compound Injected	No. Larvae	Time,* hr	No. Chromosomes		Nonpuff 60C-F	Puff Forms 60AB	Nonpuff 59A-F	Puff Present 58E-F	Nonpuff 58A-D	Average Grains per 100 μ^2
H^3-thymidine: unlabeled chromosomes	4	1/2/1/2 to 7/4	21	Grains† μ^2†	1.7 (603)	0.4 (488)	2.8 (412)	1.4 (489)		0.3
H^3-thymidine: labeled chromosomes	3		13	Grains μ^2	13.5 (321)	4.8 (248)	20.9 (233)	0.5 (112)	25.2 (162)	6.0
H^3-cytidine:	3	2/2 to 2 1/2/3	23	Grains μ^2	69.2 (588)	144.4 (673)	33.3 (477)	94.6 (409)	37.0 (240)	16.1

* Number of hours from injection of precursor to puparium formation/number of hours from puparium formation to fixation of salivary glands.

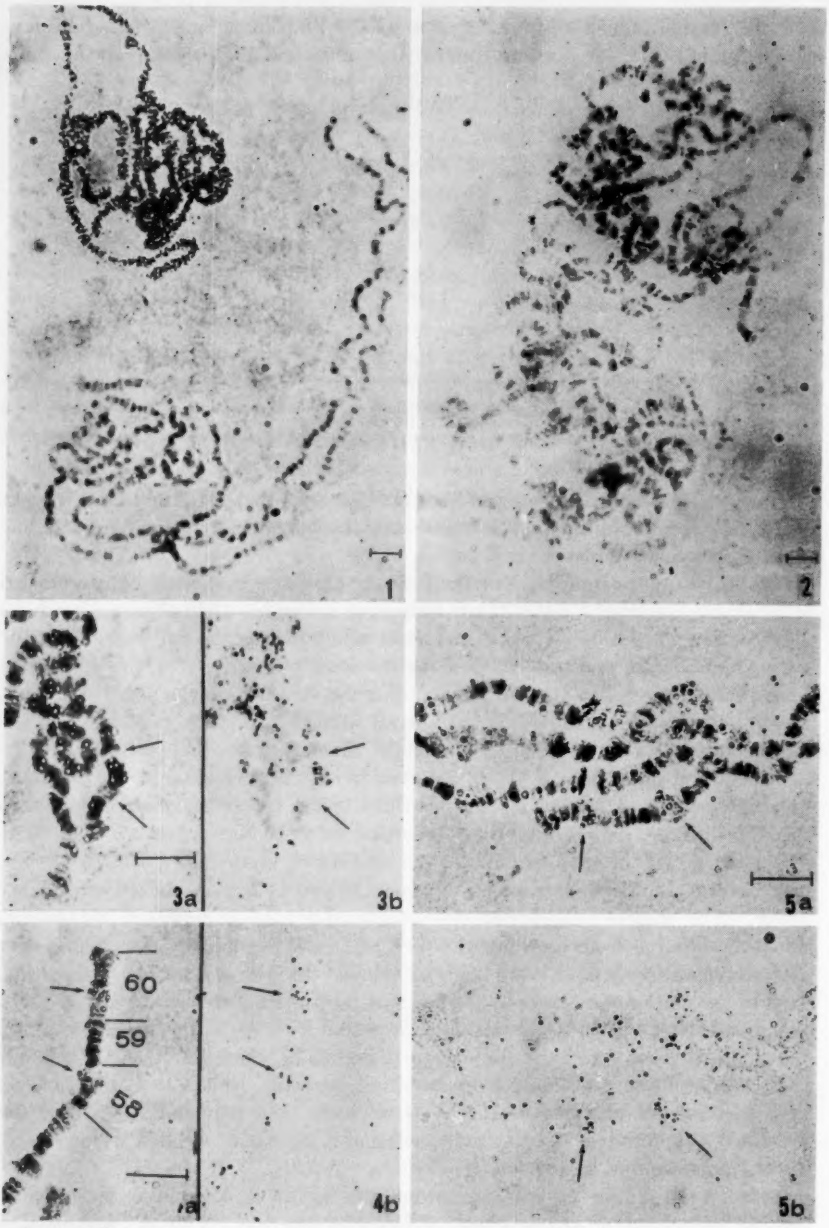
† The upper figure is the sum of the number of grains above background for all of the chromosomes; the lower figure (in parentheses) is the sum of the areas of all of the chromosomes.

One month exposure to stripping film except one week for three "unlabeled" and one "labeled" chromosome in the thymidine injected series.

typical in early prepupae, the conclusion can be drawn that DNA synthesis is not a necessary concomitant of puff formation in *Drosophila*, nor need it occur while either of the regions under study is puffed.

The distribution of grains over the few chromosomes that were lightly labeled by H^3-thymidine (Fig. 3, Table 1) can be used to determine whether or not synthesis of DNA, when it did occur, was disproprotionately high in the puff region. If the grain count for the puff and nonpuff regions were proportional to the amount of DNA in the same regions before puffing, then the synthesis of the new DNA could be said to be "proportionate"; if not, then it would be "disporportionate" and the regions in which relatively larger or smaller amounts had been formed could be identified by inspection. We have observed in unpublished studies[26] on the ultraviolet absorption of section 60 that in third instar larvae just before puparium formation, 61 per cent of the total absorbancy of the section (at 257 mμ) is in subsection 60C-F or the nonpuff region. Since we observed 18.3 (13.5 + 4.8) grains over the entire section we would expect to find 11.2 grains for the subsections 60C-F. The observed (13.5:4.8) and calculated (11.2:7.1) numbers are not significantly different from each other ($0.3>P>0.2$) and the negative deviation is over the puff region (see below). It may be concluded that the amount of DNA synthesized in the chromosome regions studied was proportional to the amount of DNA already present. This is the result that would be expected from the classical picture of chromosome duplication under a regime in which the "DNA constancy rule" was in effect. It should be pointed out, however, that data from relatively large segments of chromosomes such as we have used here do not exclude the possibility that different results might be found if the bands within a segment were studied individually.

Different intensities of labeling among the nuclei of a gland most probably indicate asynchrony in the synthesis of DNA by the cells. During the time that the isotope was available the chromosomes without autoradiographs were not undergoing synthesis, those with weak autoradiographs had just begun or were just finishing a duplication cycle and incorporated isotope during a small fraction of



The scale lines (above figure numbers) represent ten microns. All figures are interference photomicrographs of autoradiographs of salivary gland chromosomes from prepupae of *Drosophila melanogaster*.

Continued on p. 1001

the treatment time, and, finally, the ones with very dense autoradiographs carried out synthetic activity during the largest fraction of the time. In a study of the short term uptake of H^3-thymidine from the food, Taylor observed similar asynchrony in the polytene nuclei of several tissues of *Drosophila repleta* during the third (last) larval instar.[27] Detailed study of the timing of the synthesis of new DNA in these chromosomes has not yet been carried out. However, the data at hand are sufficient to show that disproportionate synthesis of DNA does not occur during formation of the puff.

Tritium administered in cytidine formed a very different pattern of silver grains. Whereas H^3-thymidine labeled exclusively the chromosomes of about 15 per cent of the nuclei, H^3-cytidine labeled cytoplasm, chromosomes and nucleoli (in increasing order of density of autoradiographic image) of all cells. It was obvious that the puff regions were relatively much more heavily labeled than they were when thymidine was administered (Figs. 4, 5). The distribution of grains (Table 1) over the four regions under study is statistically different from the distribution observed in thymidine injected larvae ($P<0.001$) and from the distribution that would be expected if the number of grains were proportional to the area of chromosome segment ($P<0.001$). In contrast to the result with thymidine, the grains are not distributed between the puff and nonpuff portions of section 60 in proportion to the ultraviolet absorbancies of the two regions ($P<0.001$); there is an excess of grains over the puff region after treatment with H^3-cytidine.

Since we have excluded DNA synthesis as a characteristic of the puffing process in section 60, incorporation of cytidine is most easily interpreted to indicate the presence of RNA. The alternative hypothesis, that the label from the cytidine is held in a kind of DNA in which cytidylic acid but not thymidylic acid derivatives turn over seems remote at the present time, but is, nonetheless, under test.[28] Since the relative total UV absorbancy changes little in the period covered, net disproportionate synthesis of RNA is unlikely, and the chromosomal label probably results from turnover of molecular units at least as large as cytosine. It is not excluded that RNA had been accumulated by the puff region at an earlier stage for it is known that radioactive phosphorus is incorporated into RNA in late third instar larvae.[14, 15]

Indeed, if an appreciable proportion of absorption at 257 millimicra in the puff were by RNA, then the proportion of grains from H^3-thymidine label should have

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FIGS. 1–3.—Tritiated thymidine injected into a larva one-half hour before puparium formation, the prepupa dissected one-half hour after puparium formation; autoradiograph exposed thirty days.

FIG. 1.—Heavily labelled nucleus above, unlabelled nucleus below. (40× double focus objective, 10× ocular.)

FIG. 2.—Lightly labelled nucleus above, unlabelled nucleus below. (Same optics as for Fig. 1.)

FIG. 3.—The tip of chromosome 2R in one of the lightly labelled nuclei. The photomicrographs labelled "a" were taken with the polarizer of the interference microscope in and with the microscope focused on the chromosome, those labelled "b" were taken with the polarizer out and the microscope focused on silver grains in the emulsion above the chromosomes. The positions of the puff regions are indicated by the arrows (see Fig. 4a); the lower one is in section 60. Note the absence of grains over the puff regions. (100× double focus objective, 10× ocular.)

FIGS. 4 and 5.—Tritiated cytidine injected into a larva 2 hours before puparium formation, the prepupa dissected three hours after puparium formation; autoradiograph exposed thirty-eight days. The tip of chromosome 2R is included in each figure: the three terminal sections upon which grain counts were carried out are marked in Fig. 4a, the positions of the puffs in sections 58 and 60 are shown by arrows in all four figures. The photomicrographs labelled "a" and "b" were taken in the manner described for Fig. 3. (Same optics as in Fig. 3.)

been lower than was calculated on the basis of absorbance alone. The deviation observed (see above), though not statistically significant for the low grain numbers observed, was in that direction and, therefore, was consistent with an excess of RNA rather than DNA in the puff region. On the other hand, the greater density of the nonpuff regions (exhibited in the interference microscope) might result in the absorption of a higher fraction of the β-radiation from the tritium tracer and bias the result in the opposite direction. It will be necessary to determine the relative specific activity of the RNA in the chromosomes before and after puff formation in order to continue the analysis. It should be pointed out that the difference between the observed distributions in the chromosomes was probably minimized by the fixative (cold 45 per cent acetic acid) by which some RNA may have been extracted but in which DNA is quite insoluble.

Although the particular puffs that have been studied in *Drosophila* on the one hand and in *Rhynchosciara* on the other have quite different metabolic patterns, the differences are probably characteristic of the chromosome regions and not the species. In *Glyptotendipes*, Stich and Naylor found, side by side, one puff that accumulated DNA and another that did not[7]; they have further indicated that wide variations in the metabolism of RNA and protein as well are to be found in different puff regions.[9] Comparable variety within the genome of *Rhynchosciara*,[2, 10, 12, 29] lends support to the prevalent view that the observed differences are derived from the products of the genes active at the puff loci.

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*PARENTAL CONTROL OF POSITION-EFFECT VARIEGATION: I. PARENTAL HETEROCHROMATIN AND EXPRESSION OF THE WHITE LOCUS IN COMPOUND-X DROSOPHILA MELANOGASTER**

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In studies of the role of extra heterochromatin in position-effect variegation at the white locus,[1, 2] discrepancies between the offspring of reciprocal crosses stimulated a search for possible genetic factors controlling gene expression in the succeeding generation. Two genotypes of mothers have already been found to enhance pigmentation in the eyes of genetically identical offspring—a recessive modifier and homozygosity as compared with heterozygosity for a w^m rearrangement.[3, 4]

It is well known that the direct effect of extra Y-heterochromatin in the genome of a fly is partial restoration of the normal phenotype disturbed when a chromosomal rearrangement juxtaposes a normally euchromatic locus to interrupted heterochromatin. The experiments reported here indicate that extra Y-heterochromatin in the parental genotype also "residually" affects offspring phenotype.

Exploratory.—Variegation was due to a 15-band insertion including w^+, Dp(w^m)-264.58a, into the proximal heterochromatin of 3L. The heterochromatin content of the genotype of the test flies was augmented by a normal Y, sc^{S1}. Y^L#2 or Y^S. Y^S#2 or Y^S:y^+ bb^+ −5 (described elsewhere[2]). Males used in the experimental crosses had an attached-XY chromosome, Y^S *w y*·Y^L y^+; females had a *y w* attached-X chromosome from a recent single ancestor. In order to reduce extraneous genetic variation, the long autosomes of all stocks were rendered initially as co-isogenic as

possible by the Cy; Ubx/Xa method[2] and crosses between stocks to yield the experimental parents were such as to randomize or eliminate background genetic differences between parents being compared. Even the distal parts of the normal and Dp-bearing third chromosomes were probably nearly identical.

All test crosses were made reciprocally for the duplication. Residual effects of the paternal extra heterochromatin (Y^F) could be assessed in XY/Y sons of XY/Y^F fathers; residual effects of the maternal Y^F, in XX/Y daughters of XX/Y^F mothers. In the other progeny, the direct effect of the Y^F is confounded with the residual effect. There were thus 18 crosses (including $Y^F = Y$) distributed into four categories, indicated in Table 1. The experiment was seriated, each of the five series including for each cross at least 5 simultaneous pair matings in vials containing aliquots of a single batch of medium. Parents were transferred to fresh medium at least once.

TABLE I

PARENTAL EFFECTS OF FIVE Y-CHROMOSOMES AND SOURCE OF Dp(w^m) ON OFFSPRING EYE PIGMENTATION[a]

Parent Contributing Dp	♀ Y^F	♂ Y	Genotype of Offspring	"Y^F" of Indicated Cross: Y Mean	Y No.[b]	sc^{S1}, Y^L Mean	sc^{S1}, Y^L No.	Y^S, Y^S Mean	Y^S, Y^S No.	$Y^S; y\ ^+bb\ ^+5$ Mean	$Y^S; y\ ^+bb\ ^+5$ No.	O Mean	O No.
♀	♀	♂	XX/Y; Dp/+	0.116	156	0.150	132	0.309	251	0.250	238	0.038	43
♂	♀	♂	XX/Y; Dp/+	0.130	148	0.205	278	0.521	431	0.561	324	0.114	157
♀	♂	♀	XX/Y^F; Dp/+	0.116	156	0.082	179	0.063	213	0.125	335	0.000	0
♂	♂	♀	XX/Y^F; Dp/+	0.130	148	0.078	98	0.051	110	0.108	92	0.000	0
♀	♀	♂	XY/Y^F; Dp/+	0.388	156	0.392	95	0.463	227	0.558	159	0.000	0
♂	♀	♂	XY/Y^F; Dp/+	0.847	132	0.771	191	1.025	352	1.395	215	0.001	3
♀	♂	♀	XY/Y; Dp/+	0.388	156	0.427	231	0.360	351	0.445	346	0.281	299
♂	♂	♀	XY/Y; Dp/+	0.847	132	1.013	84	0.981	140	0.930	97	1.017	132

ORTHOGONAL ANALYSIS OF VARIANCE

Offspring Genotype	Source of Variance	D. F.	Mean Square[c]	Variance Estimate: Total	Variance Estimate: Per Cent
XY/Y^F; Dp/+ (excluding $Y^F = 0$)	Parental source of Dp	1	3.1291*	0.1537	65.4%
	Y^F in genotype	3	0.3190*	0.0264	11.2%
	Between series	4	0.1096	...	...
	Residual[d]	35	0.0550	0.0550	23.4%
XY/Y; Dp/+	Parental source of Dp	1	4.1660*	0.1658	82.3%
	Father's Y^F	4	0.0151	...	...
	Between series	4	0.0904*	0.0071	3.7%
	Residual	42	0.0193	0.0193	10.0%
XX/Y^F; Dp/+ (excluding $Y^F = 0$)	Parental source of Dp	1	0.0003	...	...
	Y^F in genotype	3	0.0097†	0.0007	21.8%
	Between series	4	0.0016	...	...
	Residual	35	0.0026	0.0026	78.2%
XX/Y; Dp/+	Parental source of Dp	1	0.2240	...	...
	Mother's Y^F	4	0.2559†	0.0218	60.7%
	Interaction between Dp-source and Y^F	4	0.0382*	0.0064	17.8%
	Between series	4	0.0231†	0.0017	4.8%
	Residual	35	0.0060	0.0060	16.7%

[a] Sum of the visually estimated amounts of pigment in both eyes.
[b] Number of flies showing any pigmentation.
[c] Asterisk indicates significance at the 1 per cent level; dagger at the 5 per cent level.
[d] The three possible two-way interactions were tested against the three-way interaction and except in the one instance noted were not significant. Their sums of squares and degrees of freedom were pooled to give the "residual" mean square.

For all offspring, the amount of pigment in each eye was estimated visually and recorded, the scale ranging from 0 for white to 1.0 for a wild-type eye. The estimates correlate highly with photofluorometric measurements after chromatography.[2]

Each mean in Table 1 is an unweighted average over the 5 series. The value for any one series was the weighted mean of all pigmented offspring when preliminary

analyses of variance indicated homogeneity between replicate pairs; otherwise, it was the unweighted mean of sib means. No transformation of scale was attempted since, though within-sib σ was correlated with m for means below 0.25 (one-eighth of the amount of pigment in a wild-type fly), σ was practically constant for larger m's. Orthogonal analyses of variance were performed on the series means.

The greatest source of phenotypic variation in sons (86 per cent in one case, 65 per cent in the other) was the parental source of Dp (w^m), there being 2.5 times as much pigment when the father rather than the mother contributed it to XY/Y sons. The paternal Y^F had no residual effect on sons. The absence of interaction between Dp-source and father's Y^F confirms the lack of divergence at possible modifying loci on the third chromosome in the Y^F stocks.

Most of the variation in XX/Y daughters (62 per cent) is attributable to the residual effect of the maternal Y^F. Thus at least part of the differences between sons with different Y^F's has the same cause. Whether sex of the parent transmitting the duplication affected average grade of pigmented daughters depended on the maternal Y^F—the increase with paternal source being least (1.1-fold) when the mother had a normal Y, greatest (3.0-fold) when she had no Y.

Resolution of Direct and Parental Effects.–The second experiment assessed direct as well as indirect effects of Y^S:y^+ bb^+ − 5(abbreviated as Y^5) and the normal Y, since the residual effects of mother's Y^F were observed in both XX/Y and XX/Y^5 daughters. Ten pair matings of each cross (Table 2) were made simultaneously and transferred to fresh medium three more times.

Table 2 gives both the mean grade of offspring which were pigmented and the percentage of all offspring which they constituted, theoretically 50 per cent if full penetrance and no viability differential. Pairs of mean grades were compared by t tests based on within-sib σ's according to the Cochrane and Cox method described by Snedecor.[5] When the replicate pairs were heterogeneous, the unweighted mean was used with an error computed from the excess of total variance over that attributable to within-sib variation.

The maternal heterochromatin has the same phenotypic effect on both Y- and Y^5-bearing daughters (cf. lines 1, 4, 7, 9 versus 2, 5, 8, and 10, respectively). A maternal Y^5 rather than Y more than doubles the proportion of pigmented daughters (though never over 50 per cent), all comparisons being significant at the 0.01 per cent level. The amount of pigment when present is similarly increased, no comparison failing significance at the 5 per cent level. Most interestingly, lack of any maternal Y more drastically reduces penetrance and expression when the mother carries rather than lacks Dp(w^m) (cf. lines 3 and 6).

In contrast to its residual effect as compared to the Y, the direct effect of Y^5 reduces both penetrance and pigmentation to about 65 per cent (cf. lines 1, 2, 4, and 5 versus 7, 8, 9, and 10). All these comparisons are consistent though not all statistically significant. Background stock differences between this and the preliminary experiment may account for the lesser direct pigment enhancement by the Y^5 here.

Independence of the direct and maternal effects of the Y^5 on penetrance when incomplete is shown by a low interaction $\chi^2 = 0.999$ $(0.30 < P < 0.35)$.

A paternal rather than maternal source of Dp(w^m) more than doubles the proportion of pigmented daughters (e.g., cf. lines 1 and 4), although as before the *amount*

TABLE 2

RESULTS OF FURTHER CROSSES EMPLOYING Y, Y^s:y^+ bb^+ — 5 AND NO FREE Y CENTROMERE, VARYING PARENTAL SOURCE OF Dp(w^m)

	Y^F	Genotype of:[a] Father	Mother	Total	Per Cent with Pigment[b]	Mean Pigment ± Error	Number of Sibships
		Daughters (XX/Y^F; Dp/+)					
Y	(1)	Y; +/+	Y; Dp/+	332	13.9	0.070 ± 0.017	4
	(2)	Y; +/+	Y^5; Dp/+	230	34.8	0.123 ± 0.019	2
	(3)	Y; +/+	O; Dp/+	159	1.3	0.030 ± 0.010	2[c]
	(4)	Y; Dp/+	Y; +/+	458	34.7*	0.073 ± 0.009	8
	(5)	Y; Dp/+	Y^5; +/+	794	49.5	0.219 ± 0.037	10
	(6)	Y; Dp/+	O; +/+	446	36.6*	0.051 ± 0.005	8
Y^5	(7)	Y^5; +/+	Y; Dp/+	494	8.7	0.039 ± 0.008	5
	(8)	Y^5; +/+	Y^5; Dp/+	1,034	18.8	0.118 ± 0.020	9
	(9)	Y^5; Dp/+	Y; +/+	516	22.3†	0.049 ± 0.005	9
	(10)	Y^5; Dp/+	Y^5; +/+	661	44.9	0.140 ± 0.042	7
				159	27.0	0.074 ± 0.017	2
	(11)	Y^5; Dp/+	O; +/+	633	25.6*	0.038 ± 0.003	10
O	(12)	O; +/+	Y; Dp/+	678	0.0	...	8
	(13)	O; +/+	Y^5; Dp/+	493	0.0	...	6
	(14)	O; Dp/+	Y; +/+	261	0.0	...	4
		Sons (XY/Y^F; Dp/+)					
Y	(15)	Y; +/+	Y; Dp/+	325	28.6	0.166 ± 0.025	4
	(16)	Y^5; +/+	Y; Dp/+	438	30.8	0.135 ± 0.022	5
	(17)	O; +/+	Y; Dp/+	609	23.6*	0.077 ± 0.010	8
	(18)	Y; Dp/+	Y; +/+	378	49.2	0.520 ± 0.192	6
	(19)	Y^5; Dp/+	Y; +/+	445	54.4	0.535 ± 0.093	8
	(20)	O; Dp/+	Y; +/+	258	48.1	0.568 ± 0.196	4
Y^5	(21)	Y; +/+	Y^5; Dp/+	181	39.8	0.241 ± 0.104	2
	(22)	Y^5; +/+	Y^5; Dp/+	893	40.3	0.248 ± 0.128	9
	(23)	O; +/+	Y^5; Dp/+	491	32.4*	0.198 ± 0.028	6
	(24)	Y; Dp/+	Y^5; +/+	687	56.9	0.960 ± 0.114	10
	(25)	Y^5; Dp/+	Y^5; +/+	712	48.7*	1.000 ± 0.236	9
O	(26)	Y; +/+	O; Dp/+	146	0.0	...	2[c]
	(27)	Y; Dp/+	O; +/+	483	0.0	...	8
	(28)	Y^5; Dp/+	O; +/+	664	0.4	0.005 ± 0.002	10

[a] The crosses from which these parents came all contain the XY and XX derived initially from one stock used in the experiments recorded in Table 1, the XY/O/XX stock. Several different compound-X chromosomes had been used in males of stocks crossed to yield the parents of Table 1.

[b] Asterisk indicates significant inhomogeneity of frequency of white versus variegated flies between sibships at the 5 per cent level; dagger at the 1 per cent level.

[c] Since the Dp-bearing mothers cannot be distinguished phenotypically from their +-bearing sisters, 18 such were used. Only if w^m daughters appear can the mother's genotype be established. Since these two progenies had 1 w^m daughter apiece, it is likely that other Dp-bearing mothers escaped detection.

of pigment, when present, is greater only in Y-bearing daughters of Y^5 mothers (cf. lines 2 and 5).

Examination of the phenotypes of the sons indicates that the Dp-source effects and the direct and residual maternal effects of extra Y-heterochromatin are of the same magnitude as in daughters, making due allowance for the 50 per cent upper limit on proportion pigmented. With a maternal Dp(w^m), XY/Y^5 sons include 1.3 times as many pigmented flies as XY/Y sons (cf. lines 15 and 21), a ratio not unexpected, given the direct (0.65-fold) and residual (2-fold) effects inferred from their sisters. The amount of pigment when present is roughly quadrupled when the Dp(w^m) was paternal rather than maternal (cf. lines 15 versus 18, 16 versus 19, 21 versus 24, and 22 versus 25), and is increased 1.8-fold by Y^5, as compared to Y (cf. lines 15 versus 21, 16 versus 22, 18 versus 24, and 19 versus 25). Although there appears to be a significant residual effect of the paternal Y or Y^5, it occurs only when the Dp(w^m) was maternal and was not established by the earlier experiment.

Testcrosses of the above progeny have indicated no persistent (i.e., "grand-

parental") effect of the various Y^{F}'s. They do suggest a phenotypic influence of the sex of the grandparent from which the Dp(w^m) was inherited, an influence not attributable to paramutation such as occurs at the R locus in maize.[6] Further investigation is required to clarify the mechanism of this influence.

Any factor which increases pigment in Dp-bearing flies may operate either (1) locally to palliate the inhibition of normal gene action in the vicinity of disturbed proximal heterochromatin or (2) more efficiently to employ in pigment synthesis whatever the w^+ gene still manages to produce. The direct phenotypic effect of any kind of Y as compared to its total absence may be either or both of these. Another such factor must be a normal product of the Y^5 fragment which persists in the egg after the maturation divisions, since the maternal effect of the Y^5 as compared to the Y is as great when the mother lacks Dp(w^m) as when she possesses it. In the presence of Dp(w^m), there is an additional residual maternal effect of both the normal Y and the Y^5 in contrast to total lack of the Y. One of many possible explanations might be that the impaired activity of the abnormally situated w^+ gene evokes substances inhibitory to pigment formation which accumulate in the cytoplasm of the maturing oöcyte. Since both the Y and Y^5 partially restore normal w^+ activity, they would lessen this accumulation. This hypothesis has the merit of explaining also the greater efficacy of the w^+ gene in the rearrangement when introduced by sperm, but requires test. In any case, the earliest detectable phenotypic effect of these oöplasmic substances occurs at the time of pigment synthesis in the pupa.

Summary.—The amount of heterochromatin in the genome has long been known to influence directly the extent of somatic variegation induced by a position-effect chromosomal rearrangement in *Drosophila*. It has now been shown that the extent of pigmentation in white-variegated eyes does not depend solely on this direct effect of heterochromatin but also is clearly enhanced if: (1) the maternal genome contains certain Y-chromosome fragments, and (2) if the rearrangement responsible for the position-effect variegation is paternal, as compared with maternal, in origin. From the standpoint of the genetic control of pigment differentiation, the novel feature displayed here is that the parental genotype can influence an ontogenetic process in the offspring that takes place rather late in the time sequence leading to an imago.

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THE SOMATIC CHROMOSOMAL CONSTITUTION OF SOME HUMAN SUBJECTS WITH GENETIC DEFECTS

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Development of techniques for reliable cultivation *in vitro* of cells from any individual under conditions of constancy of karyotype, made possible routine establishment of normal diploid cell strains taken from human subjects with known or suspected genetic aberrations.[1] The availability of a simple, precise method for delineation of the chromosomes of such cells grown as monolayers on glass,[2] and completion of the morphological characterization of all the human chromosomes.[3] also made possible analysis of such cells for cytogenetic abnormalities. Several individuals with genetic diseases have now been screened for relatively gross chromosomal abnormalities. Aberrations of the sex chromosomes offer particularly simple applications of such analysis because of the great difference in size between the human X and Y chromosomes.[3] An independent program of somatic chromosomal analysis has also been undertaken by several British investigators, utilizing bone marrow biopsies, in which mitotic figures of dividing cells are examined directly with few or no divisions occurring *in vitro*.[7, 10a]

Methods.—Stable, *in vitro* cultures were initiated from skin biopsies of about 10–30 mgs taken from the forearm or back of the neck, as previously described. The cells were dispersed by trypsin, plated, and incubated, in the standard manner.[1] Human cord serum, rather than fetal calf serum, was employed as a nutritional supplement, since it appeared to give somewhat better results than those obtained with the particular batches of fetal calf serum available to us during the preceding winter. As an economy measure, the concentration of N16 solution in the farming medium was dropped from 40 per cent to 20 per cent, with no adverse effect on the results. Within 3 weeks of cultivation, cells form a confluent layer on the petri dish, and may be trypsinized, transferred to culture bottles, and farmed continuously, as indicated.[1] After sufficient cells had been grown for chromosomal analysis, the cultures were frozen and stored at −70°C by the procedure of Swim *et al.*[4] Such cultures, on re-thawing after periods of several months, have grown excellently and revealed an unchanged chromosome constitution. Samples taken for chromosome delineation were treated as described earlier,[2] except that after incubation for 1 hr with colchicine (0.15 γ/cm^3) the cells were placed at room temperature for 15 min, then flooded with 0.064 per cent NaCl (adjusted to pH with 10^{-3} *M* phosphate buffer) at room temperature in which solution they remained for 10 min for the hypotonic swelling step originally introduced by Hsu.[5] Fixation was accomplished by consecutive 10-sec immersion of the slides into dilutions of the standard fixative (alcohol:acetic acid:formalin = 6:2:1) of 1:2000, 1:1000, 1:50, followed by a 30-sec immersion into full strength fixative. The slides were then rinsed in water, air-dried and stained, exactly as previously described.[2] The same fixation and staining procedure was employed for the Barr sex chromatin determination on both tissue culture cells or buccal smears, except that treatment with colchicine and hypotonic saline was omitted. Photographs were taken with a Leitz Ortholux Aristophot camera-microscope.

Results.—Earlier reports have demonstrated the highly uniform chromosomal constitution which is regularly obtained by the methods here employed from the somatic cells of different normal human subjects, regardless of the organ which is sampled.[2, 3] For comparison with the new karyotypes here to be presented, the typical karyotype of the human male cell, as obtained from normal subjects, is

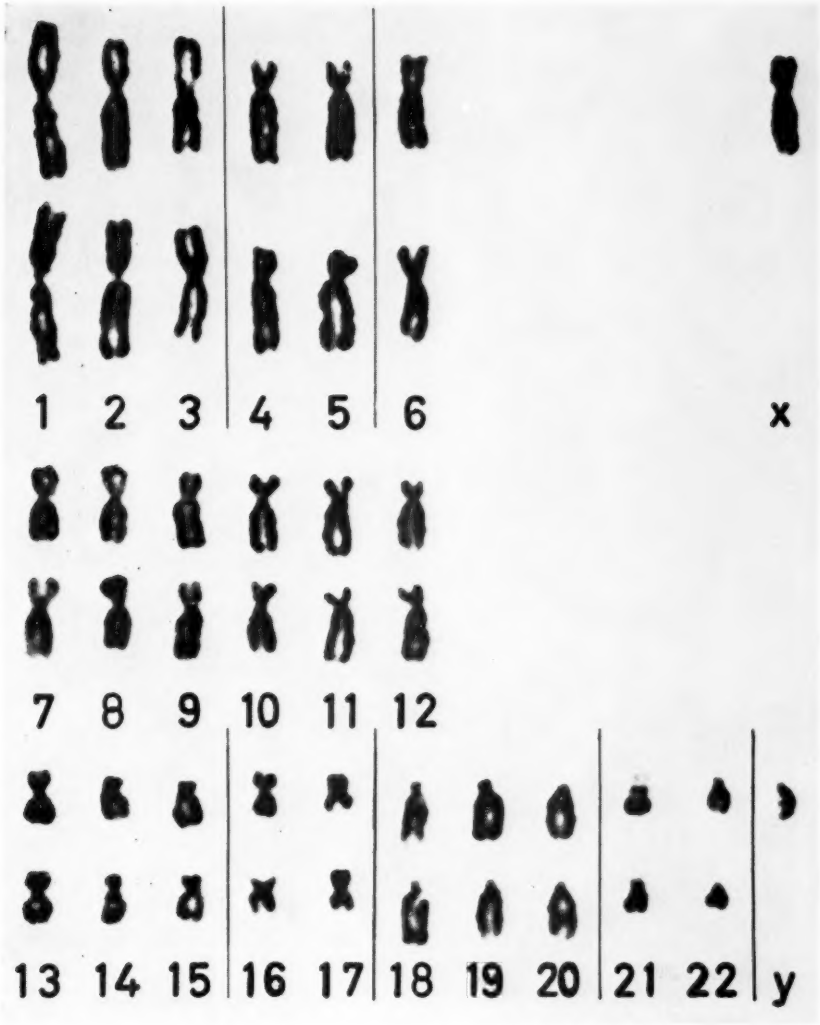

FIG. 1.—The ideogram of a normal human male. The normal female is the same, except for its possession of an additional X and no Y chromosome (3).

FIG. 2.—A typical chromosome set from the patient with ovarian dysgenesis. Only 45 chromosomes are present.

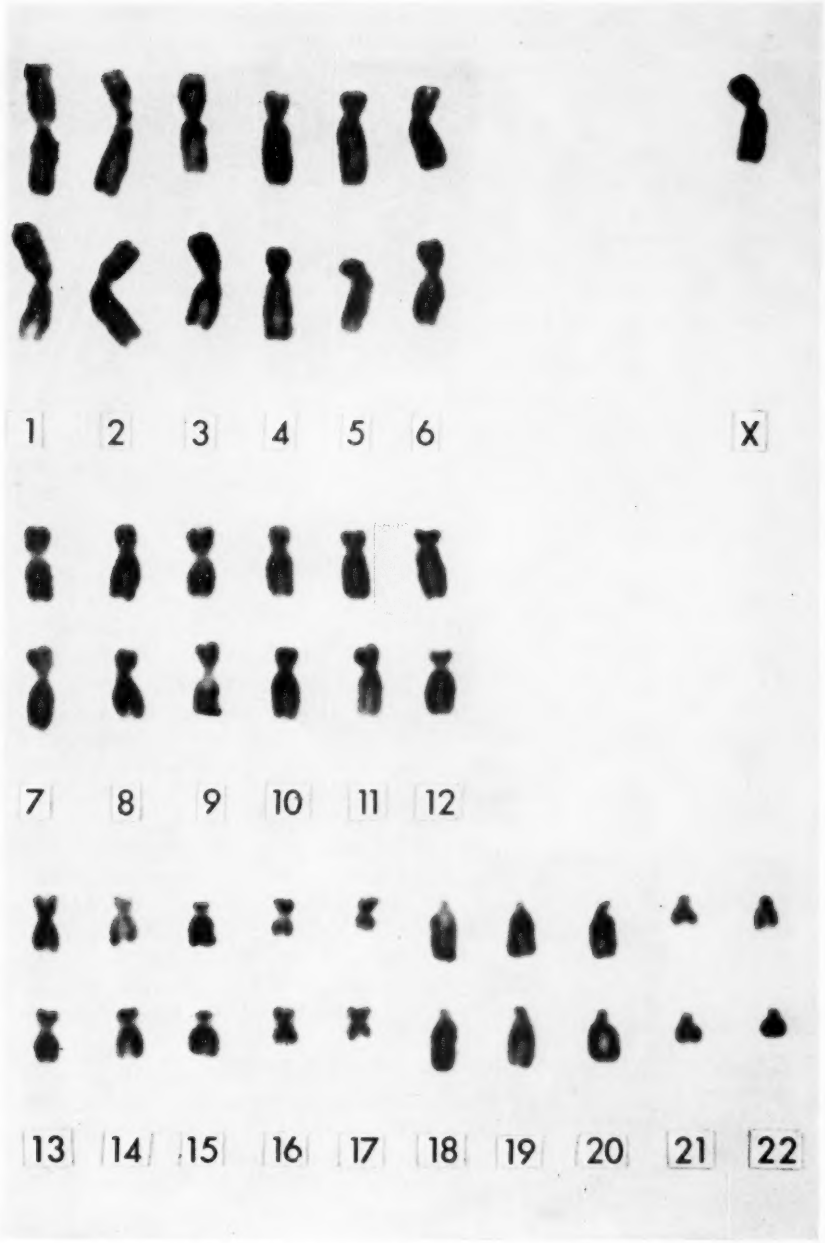

FIG. 3.—Identification of the chromosomes from the patient with ovarian dysgenesis. The numbers refer to the Tjio and Puck[3] classification of the human chromosomes.

FIG. 4.—Normal complement of 46 chromosomes from a female patient with Gaucher's Disease.

reprinted in Figure 1. The normal female karyotype is identical, except for the possession of an additional X and no Y chromosome.

Chromosomal analysis of the somatic cells of a female patient with gonadal dysgenesis was carried out.† Diagnosis of gonadal dysgenesis of this 17-year-old female was made on the basis of shortness of stature; primary amenorrhea; impalpable condition of the ovaries; female genitalia; mammary hypoplasia; small uterus; negative Barr test for female sex chromatin; and the characteristic, elevated urinary excretion of gonadotropins. Webbing of the neck was absent, but the typical reverse direction of the whorls of the hair on the back of the neck was present. Other abnormalities like heart defects, cubitus valgus, and mental retardation sometimes associated with this disease were absent.

In Figure 2 is presented a typical set of chromosomes from this patient, revealing the presence of only 45 members. Analysis of more than 150 clearly delineated mitotic figures revealed no other chromosome number, except for an occasional tetraploid cell with 90 chromosomes. In Figure 3, the chromosomes have been identified and labeled in accordance with the classification system of Tjio and Puck.[3] It is evident that the autosomes are normal, but only one sex chromosome, the X, is present.

Single cells from the culture of the XO individual, when plated by the standard technique,[1] readily formed colonies in the medium here employed, with plating efficiencies like those of other human cell lines. The growth rate is approximately the same as that of normally diploid male or female human cells. Hence, in our medium, no growth hindrance is caused by the missing chromosome. Other biochemical studies are in progress.

In contrast to these findings, the chromosomes of skin cells of patients with Marfan's syndrome, phenyl ketonuria, and female pseudohermaphroditism‡ and of the bone marrow cells of a patient with Gaucher's disease have been found to be normal with respect to number and gross morphology. Figure 4 presents a picture of the chromosomes of the female patient with Gaucher's disease.

Detailed measurement of individual chromosome arms in all these cases, to determine whether minute deletions or other abnormalities exist, is in progress and will be reported elsewhere.

Discussion.—Study of several thousand randomly selected mitotic figures obtained by the technique here employed from somatic cells of presumably normal human subjects has yielded the normal chromosome number (46 or, in an occasional cell, the tetraploid 92) with a constancy better than 99.9 per cent. Though far fewer cells of normal persons have as yet been accurately measured for chromosomal morphology, our data have shown remarkable anatomic constancy of the individual chromosomes in the cells of presumedly normal persons except for occasional individuals with more pronounced satellites in 1 or more chromosomes of the pairs 18 or 21.[2] A similar degree of constancy of human chromosomal morphology has been reported by Chu and Giles.[6] Thus, application of these methodologies to cells of persons with known or suspected genetic defects can be carried out with considerable confidence.

The present results demonstrate that the absence of the Barr sex chromatin reaction is not sufficient grounds for concluding a male chromosomal constitution to exist in the given cell type. While the simplicity of the Barr test is a great ad-

vantage, it does not afford as much information as does delineation of the entire karyotype.

These findings make it evident that a human subject with an XO sex chromosome constitution is female rather than male, though femaleness is not complete, since the ovulation function is absent. The present data demonstrates that in Man the Y chromosome contains genes needed for development of both the primary and secondary male characteristics, a condition very different from that in *Drosophila*. Similarly, the fact that a human XO subject is not possessed of the complete apparatus for ovulation suggests that the double complement of X chromosomal genes is necessary in an autosomally diploid individual for full expression of normal female reproductive power. Since the determinants for maleness resides to so large an extent in the Y chromosome, it is conceivable that true human hermaphroditism might arise as a result of exchange between the X and Y chromosomes, or from simple deletion in the Y chromosome, as well as from the production of the genetic constitution XXY, as has been recently reported as in a case of Klinefelter's syndrome.[7] In this case, however, the occurrence of XXY human females inferred by Lewit and Serebrowsky[8] from the data of Cunier[9] would require re-interpretation.

It is evident that the chromosome loss associated with ovarian dysgenesis in the patient here studied could have resulted from non-disjunction in the stemline of the egg, which caused loss of its X chromosome, or in that of the fertilizing sperm, causing loss of either its X or its Y chromosome. The possibility of a mosaicism developing after fertilization cannot yet be excluded, although it seems unlikely and could be checked by sampling cells of tissues with maximally divergent embryologic development. Of particular interest will be the study of cells of individuals with ovarian dysgenesis accompanied by a positive sex chromatin reaction, a condition which could arise from a deletion in one X chromosome.

Non-disjunction in the sex-chromosomes of the sperm could lead to individuals with either XXY or XO constitution, while if it occurred in the egg, it would lead on fertilization to XXX, XXY, XO or YO constitution of which the last is probably lethal. Thus, if non-disjunction in gametogenesis is the principle cause of these conditions, Turner's and Klinefelter's syndromes should occur with equal frequencies, while the XXX condition, not yet described for man, should occur at a lower rate. Study of these frequencies and search for the superfemale, XXX condition among precociously pubescent females is in progress.

As the present paper was being written, an independent series of papers[10] appeared in the *Lancet* by Ford, Jacobs and their co-workers, describing further developments in their highly productive program, analyzing the chromosomal constitution of somatic (bone marrow) cells from human patients with various genetic diseases, and including a study of the chromosomes of a case of Turner's syndrome. The chromosomal analysis of that patient is identical to the one presented here, in revealing an XO constitution, despite some differences in some of the clinical aspects, as in the subnormal mentality of the English patient, in contrast to the far-above-average intelligence of our own.

In another study which has also just appeared,[11] Russell and his co-workers report the occurrence in mice of individuals with sex chromosome constitution XO, which possess the anatomy of a female. Such animals were able to carry out normal female reproduction. However, the presence of a particular sex-linked gene

in some such individuals made reproduction possible only when their ovaries were transplanted to a normal female mouse. These investigators concluded that, for the mouse, maleness requires the Y chromosome. These findings lead to the expectation that in human females too, the XO chromosome condition may display different kinds of reproductive behavior depending on the individual's genic constitution. Moreover, the fact that in the mouse, at least some XO karyotypes can ovulate normally leads to the speculation that XO human females might be induced to develop more completely if the molecular (and presumably hormonal) environment were properly adjusted during prenatal or possibly postnatal development. In principle, at least, it is possible by means of the present technique to diagnose such conditions in the foetus. While the many different kinds of complexities involved are fully appreciated, it does not seem impossible that, as in one or two other conditions, ultimately it may be possible to prevent some of the pathologic consequences of this genetic defect (though not the defect itself) in humans.

The differences in the frequency of occurrence of the associated clinical symptoms in patients with ovarian dysgenesis who display the same chromosomal constitution may involve: the existence of allelic differences in the genes of the remaining X chromosome, (such genes, however, would normally have to be present on the Y chromosome also, since these manifestations are not frequent in males); the development of complex interactions of biochemical imbalance due to the XO condition with subtle gene defects on other chromosomes; or, if mosaicism is involved, differences in the time and site at which the abnormal chromosomal condition arose.

It is obvious that the XO cells here described furnish a genetically labeled strain which offers possibilities for a variety of experimental operations.

The methods here employed for chromosomal analysis of the somatic cells require more time than those utilizing bone marrow biopsy. However, they are less painful for the patient and permit even repeated sampling of patients because of the ease of removal of the tiny amounts of skin required. Moreover, they also make possible establishment of the cells in stable, continuous, *in vitro* growth for further genetic and metabolic studies.

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† A report on this case was presented at the Meeting of the Society for Pediatric Research, at Buck Hill Falls, Pa., May 6, 1959.

‡ This patient is an infant, 2 months of age, with male-appearing genitalia consisting of a phallus with first degree hypospodias, no scrotal masses, hirsutism, and a positive sex chromatin test. The child developed Addisonian-like crises which responded to steroids, DOCA, and salt. An older sibling died in infancy with a similar condition.

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*THE EFFECTS OF DELETIONS, POINT MUTATIONS, REVERSIONS AND SUPPRESSOR MUTATIONS ON THE TWO COMPONENTS OF THE TRYPTOPHAN SYNTHETASE OF ESCHERICHIA COLI**

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Introduction.—In previous investigations with the tryptophan synthetase (TSase) of *Neurospora crassa*[1] and *Escherichia coli*[2] it was observed that gene mutations affecting the formation of this enzyme may result in the production of a protein (designated CRM) which is immunologically similar to the enzyme. In other mutants lacking TSase, in both organisms, this protein was not detected. Subsequent studies have shown that the TSase of *E. coli*[3, 4] and *N. crassa*[5] catalyzes two reactions (2 and 3) in addition to the previously studied reaction, (1).

(1) indole + *L*-serine → *L*-tryptophan
(2) indoleglycerol phosphate ⇌ indole + triose phosphate
(3) indoleglycerol phosphate + *L*-serine → *L*-tryptophan + triose phosphate

It was also concluded that reaction (3) is not the sum of reactions (1) and (2) since free indole is not formed during the course of this reaction. It appears likely therefore that reaction (3) constitutes the actual physiological mechanism of tryptophan synthesis in these organisms. Investigations on the identity of the CRM's formed by several of the TSase-lacking mutants of *E. coli* which were previously studied revealed that these proteins were enzymatically active in reaction (2), but not in the other reactions.[6] Thus the CRM's in these strains appear to be altered proteins which have retained both an enzymatic activity and an immunological identity characteristic of wild type TSase. More recent studies[4] with the TSase of *E. coli* have led to the finding that this enzyme system consists of two separable protein components (designated A and B), both of which are required for the catalysis of any of the above reactions at maximal rates. Each of the components, when tested separately, appears to have slight activity in one of the three reactions—component A in reaction (2), and component B in reaction (1). However, in neither case is the activity more than ten per cent of that obtained with a mixture of the two components.

In view of this new information on the two-component nature of *E. coli* TSase it was of interest to examine the various strains of *E. coli* in which TSase activity has been affected by mutation. The present report is concerned with the results of this investigation.

Materials and Methods.—All the tryptophan auxotrophs studied except the three "deletion" types were isolated by the penicillin method following ultraviolet irradiation of a culture of *Escherichia coli* strain K-12. The growth requirements and accumulation behavior of these strains are summarized in Table 1. Strain T-3 is blocked at an early step in tryptophan biosynthesis; when grown on limiting amounts of anthranilic acid it forms large amounts of normal components A and B. The A and B mutants are revertible auxotrophs with defects in the formation and/or activity of the A or B protein of TSase. The strains designated B/1T7, B/1TN, and C13T are tryptophan auxotrophs of *E. coli* strain B; they acquired their tryptophan requirement simultaneously with mutation to resistance to bacteriophage T-1. These strains appear to lack different segments of the gene cluster controlling tryptophan synthesis.[7] In all three mutants the deleted loci include the A and B genes, i.e., the genes concerned with the formation of proteins A and B.

Revertants, partial revertants, and suppressed mutants were obtained from several A and B mutants following ultraviolet irradiation and selection for ability to grow in the absence of a tryptophan supplement. Partial revertants and suppressed mutants were initially distinguished from revertants on the basis of their slow growth in the absence of, and fast growth in the presence of tryptophan, and by their accumulation of either indoleglycerol or indole. Suppressed mutants were distinguished from revertants and partial revertants by transduction tests; the original mutant types were recovered from suppressed mutants but not from revertants.

Mutational site mapping: The relative linear order of the mutationally altered sites in the A and B point mutants was established using transduction with phage Plkc.[7] In these experiments the various A and B point mutants were employed as donors or recipients and the frequency of appearance of tryptophan independent colonies was determined for each donor-recipient pair. To eliminate variables such as lysis of recipient cells and efficiency of adsorption, transduction from *his*$^-$ to *his*$^+$ was scored in the same experiments; that is, the donor strains were *his*$^+$*tryp*$^-$ and the recipients were *his*$^-$*tryp*$^-$, and the number of tryptophan-independent colonies observed in plated aliquots expressed as a fraction of the number of transductions to histidine independence. Joint transduction of *his* and *tryp* does not occur.

Extract preparation: Cells were grown at 37° with shaking on a minimal medium containing 0.2 per cent glucose and sufficient anthranilic acid, indole, or tryptophan to give near-maximal growth. A supplement of 0.1–0.5 per cent acid hydrolyzed casein was included in the growth medium of mutants B-1 through B-6 and the deletion mutants to increase the yield of these bacteria. Extracts were prepared by sonic oscillation.

Enzyme assays: A detailed description of the methods used to assay TSase components A and B in reaction (1), (2), and (3) has appeared elsewhere.[4] In all cases the incubation mixture contained, in addition to the substrates, a threefold excess of the component which was not being assayed. As shown previously,

TABLE 1

EXAMINATION OF DELETION AND POINT MUTANTS

Strain	Growth Response*	Accumulation	Reaction	Enzymatic Assay: Component A S.A.§	Component A Per Cent†	Component B S.A.§	Component B Per Cent†	Immunological Assay: A-CRM, S.A.§	B-CRM, S.A.§
Wild type K-12	Min.	...	In → Tryp	2.9	100	2.7	100		..
			InGP → In	0.1	3.5	0.07	2.6	...	..
			InGP → Tryp	1.1	38	0.9	33	...	..
T-3	An, In, Tryp	...	In → Tryp	18.7	100	13.3	100	...	..
			InGP → In	0.6	3.2	0.5	3.8	...	..
			InGP → Tryp	9.0	48	6.1	46	...	..
B/IT-7	Tryp	...	In → Tryp	0	...	0	...	0	0
C-13-T	Tryp	An	InGP → In	0	...	0	...	...	..
B/IT-N	Tryp	InG	InGP → Tryp	0	...	0	...	...	..
A-1	In, Tryp	InG	In → Tryp	1.9	...	5.0	100	...‡	..
			InGP → In	0	...	0.09	1.8	...	..
			InGP → Tryp	0	...	2.1	42	1.01	..
A-2	In, Tryp	InG	In → Tryp	0	...	28.7	100	0	..
			InGP → In	0	...	0.9	3.1	...	..
			InGP → Tryp	0	...	14.1	49	...	..
A-3	In, Tryp	InG	In → Tryp	10	...	9.7	100	...‡	..
			InGP → In	0	...	0.13	1.3	...	..
			InGP → Tryp	0	...	3.1	32	5.3	..
A-4	In, Tryp	InG	In → Tryp	0	...	24.7	100	0	..
			InGP → In	0	...	0.74	3	...	..
			InGP → Tryp	0	...	11.8	48	...	..
B-1	Tryp	In	In → Tryp	32	100	0	...	...	35
			InGP → In	1.1	3.3	1.3	...	...	..
			InGP → Tryp	11	33	0	...	...	..
B-2	Tryp	In	In → Tryp	41	100	0	...	...	20
			InGP → In	1.8	4.4	1.2	...	...	..
			InGP → Tryp	16	39	0	...	...	..
B-3	Tryp	In	In → Tryp	47	100	0	...	...	20
			InGP → In	1.6	3.4	0.6	...	...	..
			InGP → Tryp	20	43	0	...	...	..
B-4	Tryp	InG	In → Tryp	6.6	100	0	...	...	0
			InGP → In	0.18	2.7	0	...	...	..
			InGP → Tryp	2.8	42	0	...	...	..
B-5	Tryp	InG	In → Tryp	11	100	0	...	...	0
			InGP → In	0.24	2.2	0	...	...	..
			InGP → Tryp	4.9	45	0	...	...	..
B-6	Tryp	InG	In → Tryp	6.4	100	0	...	...	0
			InGP → In	0.17	2.7	0	...	...	..
			InGP → Tryp	2.7	42	0	...	...	..

* An = Anthranilic acid; In = indole; InG = indoleglycerol; InGP = indoleglycerolphosphate. Tryp = tryptophan.
† Setting the In → Tryp activity at 100 per cent.
‡ Inhibited to the same extent as normal A in this reaction (by antibody).
§ S.A. = Specific activity.

maximal A or B activity is obtained with a threefold or greater excess of the other component. Partially purified component A and partially purified component B, each free of the other component, were employed as assay reagents. Reactions (1) and (3) were performed in a 1 ml. incubation mixture containing serine, pyridoxal phosphate, indole or indoleglycerol phosphate, 0.09 *M* tris buffer at pH 7.8, and 0.03 ml of a saturated solution of NaCl. Reaction (2) was assayed in 0.1 *M* phosphate buffer, pH 7.0, in the presence of 5×10^{-3} *M* hydroxylamine. Indole disappearance (reaction 1) or appearance (reaction 2) was estimated colorimetrically, while tryptophan formation (reaction 3) was determined microbiologically[8] on the supernatant of a perchloric acid-treated reaction mixture. One unit of A or B activity in reaction (1), (2), or (3) is defined as the amount which catalyzes the disappearance of 0.1 μM of reactant or the appearance of 0.1 μM of product in 20 minutes at 37° C. Specific activity is expressed as units of enzyme activity per milligram

of extract protein. Protein was determined by the method of Lowry *et al.*[9]

Immunological assays: Antisera produced in rabbits in response to injections of partially purified *E. coli* TSase preparations (containing components A and B) have been found to have high anticomponent B activity and low anticomponent A activity. The anti-A in these sera could be removed by absorption with component A. Specific antibodies to component A were obtained by the injection of a highly purified preparation of component A, employing the adjuvant technique. No immunological cross reaction between the two components was demonstrable. One unit of anti-A or anti-B serum is defined as the amount which inhibits one unit of A or B activity in any of the three reactions. The term CRM[10] (cross-reacting material) will be used to refer to an altered protein, present in a mutant extract, which can combine with antibody to its *normal* counterpart. Altered component A will be designated A-CRM and altered component B, B-CRM. The methods used for the immunological assay of B-CRM have been described previously.[6] In the tests reported here, crude anti-B serum was employed and a threefold excess of component A was included in the reaction mixture to insure that the small amount of antibody to component A present in the anti-B serum would not contribute to the observed inhibition of activity. A-CRM assays were performed in the same manner as B-CRM assays. One unit of A or B-CRM (A or B antigenic material) is defined as the amount of protein which combines with one inhibiting unit of the appropriate antiserum.

Results.—Deletion mutants: Three deletion mutants, each lacking genic material corresponding to genes A and B, have been examined for component A or component B activity in the three biochemical reactions catalyzed by these proteins. As can be seen in Table 1, no trace of activity in extracts of any of the three mutants was detected. In other tests (Table 1) these strains were examined for A-CRM or B-CRM, but neither was detected. Thus the deletion mutants appear to represent strains in which the absence of genes A and B results in the loss of ability to form components A and B, and any protein or proteins immunologically similar to these components.

Point mutations affecting components A and B: Four mutants with defects in the formation or activity of component A have been studied to date; these strains were designated A-1 through A-4. All four of these strains yielded tryptophan-independent colonies in spontaneous reversion experiments; thus they were provisionally classified as point mutants. The mutationally altered site in each of these strains was mapped and all four sites were located in one small region of genetic material. Strains A1 and A3 form an A-protein which is effective in the indole → tryptophan reaction, but not in either of the reactions involving indoleglycerol phosphate (Table 1). Mutants A2 and A4 do not form any material which exhibits component A activity in any of the three biochemical reactions. Immunological tests with extracts of the four A mutants are consistent with these findings; they indicate that mutants A2 and A4 do not form an A-CRM while mutants A1 and A3 do. Furthermore, these tests demonstrated that the A-CRM in the latter two strains, though completely inactive in reactions (2) and (3), has the same activity to antigen ratio in reaction (1) as does normal component A. All four A mutants produce normal component B (Table 1).

The four A-mutants were indistinguishable in accumulation tests; indoleglycerol

was the predominant compound detected. In growth experiments they all responded to indole as well as to tryptophan. The ability of mutants A1 and A3 to grow in the presence of indole is understandable since these strains form an A-CRM which is effective in the conversion of indole to tryptophan. The response of mutants A2 and A4 to indole cannot be explained in this manner, since these strains do not form normal or altered component A. However, they do form abnormally high levels of component B (see Table 1), and the activity of this component in reaction (1) (in the absence of component A) is probably sufficient to account for their growth behavior. This activity is about 1–3 per cent of the activity exhibited in the presence of component A when assayed in tris buffer, and 5–10 per cent when assayed in phosphate buffer.

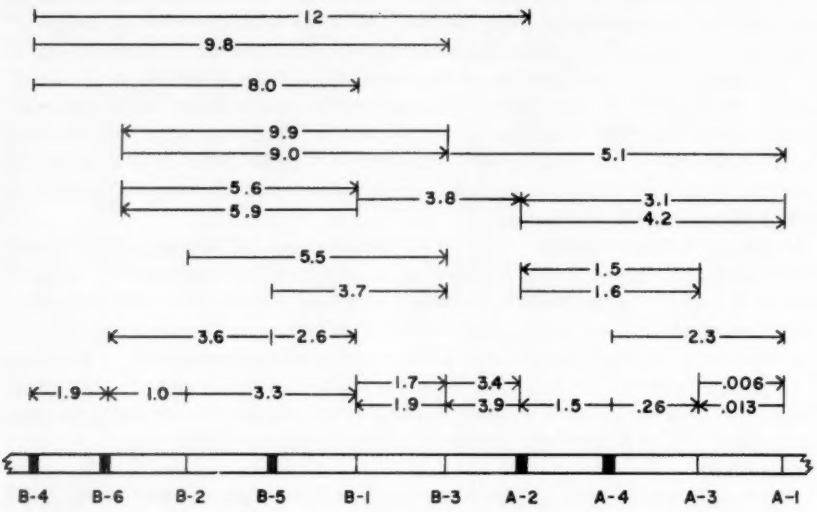

FIG. 1.—Tentative mutational site map for genes A and B. Black bars indicate the location of mutationally altered sites which lead to inability to form the A or B protein. The thin lines represent mutationally altered sites in A-CRM or B-CRM producing mutants. The arrows indicate direction of transfer.

Six auxotrophs in which component B has been affected by mutation have been examined in biochemical, immunological, and genetic tests. All six of these mutants can revert. The mutationally altered site in each of these strains has been mapped and found to lie within a small region of genetic material. Of these six B auxotrophs three accumulate indole and three accumulate indoleglycerol. The indole accumulators, on enzymatic examination, were found to form a B protein which is active in reaction (2) but ineffective in reactions (1) and (3) (Table 1). Extracts of the other three B mutants, the indoleglycerol accumulators, do not have component B activity in any of the three biochemical reactions. Immunological tests performed with extracts of the six B mutants indicate that those strains which have indoleglycerol phosphate → indole activity form a B-CRM while the other

three B mutants do not. The B-CRM formed by two of the mutants is as effective per unit B-antigenic material in reaction (2) as is normal component B (Table 1). The B-CRM in the third mutant, strain B-2, is more active enzymatically per unit B-antigenic material than is normal component B. All six of the B mutants form component A which appears normal in the three biochemical reactions.

Mutational site map for A and B point mutants: The results of the transductional analyses designed to map the mutationally altered sites in the A and B point mutants are summarized in Table 2, and the mutational site map based on these data is shown in Figure 1. It can be seen that the data obtained permit the arrangement of the altered mutational sites in a reasonable linear order. Agreement between duplicate experiments was very poor, however; sometimes the values obtained varied by as much as 40 per cent. Mutants A-3 and A-4 gave an unexpectedly small number of *tryp*$^+$ colonies in reciprocal transduction experiments. The other transductions with these strains indicated that much higher values should have been obtained. The explanation for this observation is not known.

TABLE 2

TRANSDUCTION EXPERIMENTS WITH A AND B POINT MUTANTS

	Recipient								
	his$^-$ *A-1*			*his*$^-$ *A-2*			*his*$^-$ *A-3*		
Donor	tryp$^+$ *	his$^+$	tryp$^+$/his$^+$, Per Cent	tryp$^+$	his$^+$	tryp$^+$/his$^+$, Per Cent	tryp$^+$	his$^+$	tryp$^+$/his$^+$, Per Cent
his$^+$ *A-1*	0	*ca.* 5,000	...	1,169	38,030	3.1	2	15,038	0.013
his$^+$ *A-2*	1,313	31,376	4.2	0	*ca.* 5,000	...	477	29,812	1.6
his$^+$ *A-3*	2	33,402	0.006	597	39,390	1.5	0	*ca.* 5,000	...
his$^+$ *A-4*	897	38,722	2.3	548	37,130	1.5	76	29,832	0.26
his$^+$ *B-1*	...	...	...	109	2,890	3.8	...	...	...
his$^+$ *B-3*	309	6,100	5.1	788	23,228	3.4	...	...	...
his$^+$ *B-4*	...	...	...	256	2,180	12	...	...	...

	Recipient								
	his$^-$ *B-6*			*his*$^-$ *B-1*			*his*$^-$ *B-3*		
Donor	tryp$^+$	his$^+$	tryp$^+$/his$^+$, Per Cent	tryp$^+$	his$^+$	tryp$^+$/his$^+$, Per Cent	tryp$^+$	his$^+$	tryp$^+$/his$^+$, Per Cent
his$^+$ *B-1*	1,711	29,233	5.9	0	*ca.* 5,000	...	251	14,959	1.7
his$^+$ *B-2*	177	16,990	1.04	403	12,358	3.3	996	18,239	5.5
his$^+$ *B-3*	759	7,665	9.9	55	2,978	1.9	0	*ca.* 5,000	...
his$^+$ *B-4*	308	16,040	1.9	897	11,280	8	2,032	20,715	9.8
his$^+$ *B-5*	304	8,574	3.6	222	8,418	2.6	364	9,959	3.7
his$^+$ *B-6*	0	*ca.* 5,000	...	584	10,439	5.6	1,095	12,134	9
his$^+$ *A-2*	...	...	...	...	...	...	932	23,816	3.9

* The *tryp*$^+$ values listed represent actual colony counts while the *his*$^+$ values given were calculated from a ten or twenty-fold dilution which was actually plated. The values reported were corrected for any reversion in control platings.

It is interesting to note from the mutational site map that the A and B genes are very close to one another and that some mutational sites in the B gene are closer to mutational sites in the A gene than they are to other mutational sites within the B gene. Furthermore, it can be seen from the B mutational site map that mutations which lead to the two different types of protein alterations are not restricted to specific regions of the mutational site map; two sites which, when mutated, lead to loss of ability to form the B protein bracket a B-CRM producing site.

Revertants and partial revertants: Ultraviolet-induced revertants were isolated from a representative of each of the A and B mutant types (CRM producers and nonproducers). These strains were examined in enzymatic and immunological

tests to determine whether or not reversion restored a normal A or B protein. Two revertants obtained from strain A3, an A-CRM producer, formed what appears to be normal component A, i.e., this component catalyzes the three biochemical reactions at the same relative rates as wild-type A and is inhibited to the same extent by anti-A serum (Table 3). The growth of these revertants is not stimulated by tryptophan, and they do not accumulate indoleglycerol. The partial revertant (strain A-2 pr-6) obtained from the A mutant which does not form A-CRM, although capable of growing in the absence of tryptophan, is stimulated by this amino acid. In addition it accumulates indoleglycerol, a property which also distinguishes it from the wild-type strain and full revertants. The component A formed by this partial revertant is capable of participating in all three of the biochemical reactions in which normal component A participates. However, the relative activities of this component A in the various reactions differs somewhat from those observed with wild-type component A (see Table 3), suggesting that an abnormal A or a mixture of A's is formed by this strain. Immunological tests (Table 3) were consistent with these observations since they demonstrated that anti-A was much less effective in inhibiting the activity of this component A than that of normal component A.

TABLE 3

ENZYMATIC AND IMMUNOLOGICAL EXAMINATION OF EXTRACTS OF REVERTANTS AND PARTIAL REVERTANTS

		Enzymatic Assay				Immunological Assay	
		Component A		Component B		A Inhibited	B Inhibited
Strain	Reaction	S.A.†	Per Cent‡	S.A.†	Per Cent‡	Per Cent of Control§	Per Cent of Control§
Wild type K-12	In → Tryp	2.9	100	2.7	100		
	InGP → In	0.1	3.5	0.07	2.6		
	InGP → Tryp	1.1	38	0.9	33		
A-2 pr-6	In → Tryp	0.3	100	1.5	100	23	
	InGP → In	0.05	17	0.044	2.9	..	
	InGP → Tryp	0.33	110	0.6	40	..	
A-3 R-2	In → Tryp	1.7	100	1.45	100	91	
	InGP → In	0.05	2.9	0.04	2.9	..	
	InGP → Tryp	0.7	41	0.61	42	..	
A-3 R-9	In → Tryp	1.6	100	1.42	100	95	
	InGP → In	0.05	3.1	0.04	2.8	..	
	InGP → Tryp	0.71	44	0.63	44	..	
B-1 pr-3	In → Tryp	44	100	0.33	100		0*
	InGP → In	1.3	2.9	1.37	415		..
	InGP → Tryp	16.7	38	0.33	100		..
B-4 R-8	In → Tryp	2.7	100	2.14	100		94
	InGP → In	0.06	2.2	0.06	2.8		..
	InGP → Tryp	1.2	44	0.68	32		..

* This extract had a B-CRM specific activity of 53.3.
† Specific activity.
‡ Setting the In → Tryp activity at 100 per cent.
§ Control was inhibition of wild type A or B by homologous antiserum.

Of the B revertants examined, strain B-4 R-8 produced a component B which was normal, as judged by enzymatic and immunological criteria (Table 3). Strain B-1 pr-3, a partial revertant derived from a B-CRM producer, formed a B-protein which could participate in all three reactions, although its activity in two of the reactions, (1) and (3), was considerably less than normal B, per unit B-antigenic material. Its component B had a normal activity:antigen ratio in reaction (2), however.

The revertants and partial revertants described in this section do not necessarily constitute the principal types obtainable from the various A and B mutants studied.

The strains examined were ones which had previously been characterized genetically and were employed in the present study for this reason.

Suppressed A mutants: Ultraviolet induced suppressed mutants derived from the two types of A and B mutants, CRM producers and nonproducers, were also examined (Table 4). Each of these suppressed mutants accumulated indoleglycerol or indole and its growth was stimulated by tryptophan. Enzymatic tests indicated that the suppressed A mutants formed normal component B and the suppressed B mutants formed normal component A. Suppression of the A-mutant which did not produce an A-CRM yielded a strain (A-2 su-2) which formed an A-protein which was indistinguishable from normal A in the three biochemical reactions and was similar in its sensitivity to anti-A serum (Table 4). However, as can be seen in Table 4, this strain produced much less component A than component B, and this abnormal behavior may be indicative of an A-protein alteration. The component A formed by the suppressed mutant derived from the A-CRM producer, strain A-3 su-3, has abnormal activity ratios and is less active per unit A-antigen in the indoleglycerol phosphate reactions than is normal component A. It appears, therefore, that a mixture of A-proteins or an altered A-protein which differs from the A-3 component A is formed by this suppressed mutant.

TABLE 4

ENZYMATIC AND IMMUNOLOGICAL EXAMINATION OF SUPPRESSED MUTANTS

		Enzymatic Assay				Immunological Assay	
		Component A		Component B			
Strain	Reaction	S.A.†	Per Cent‡	S.A.†	Per Cent‡	A Inhibited Per Cent of Control§	B Inhibited Per Cent of Control§
A-2 su-2	In → Tryp	0.29	100	13.5	100	*ca.* 50–80	..
	InGP → In	*ca.* 0.01	3.4	0.46	3.4	...	..
	InGP → Tryp	0.12	41	6.6	49	...	..
A-3 su-3	In → Tryp	43.4	100	37.8	100	89	..
	InGP → In	*ca.* 0.005	0.01	0.5	1.3(?)	..	..
	InGP → Tryp	0.19	0.44	15.8	42	..	..
B-1 su-1	In → Tryp	53.6	100	0.36	100	..	0*
	InGP → In	1.7	3.2	1.7	470	..	..
	InGP → Tryp	23.9	45	0.28	78	..	..
B-4 su-4	In → Tryp	5.2	100	0.79	100	..	97
	InGP → In	0.1	1.9	0.013	1.6	..	..
	InGP → Tryp	1.9	37	0.36	46	..	..

* Also had a B-CRM specific activity of 59.8.
† Specific activity.
‡ Setting the In → Tryp activity at 100 per cent.
§ Control was inhibition of wild type A or B by homologous antiserum.

A suppressed mutant (strain B-1 su-1) derived from a B-mutant which does not form B-CRM was found to produce what appears to be normal component B, although, as was the case with the corresponding suppressed A-mutant, the level of B present was unusually low when compared with the level of component A which was formed. The component B formed by the suppressed mutant (B-4 su-4) derived from the B-CRM producer had abnormal activity ratios in the various reactions and was less active per unit B-antigenic material in the reactions yielding tryptophan than was normal component B. Thus this suppressed mutant probably forms a mixture of B-proteins, or a component B which differs from both the normal B and the mutant B.

Discussion.—The results obtained with the deletion mutants and the A and B point mutants indicate that specific genic regions control the formation of the A and B proteins, and that point mutations affecting one of these proteins have

no effect on the formation of the other. In the point mutants which were examined two types of mutational effects on each of the components of TSase were detected, one leading to the loss of ability to form any protein recognizable enzymatically or immunologically as component A (or B), and the other leading to the formation of an altered protein with one or more properties similar to those of the normal component. The mutations leading to these two types of defects do not appear to be entirely restricted to different segments of the gene since in one case a B-CRM mutational site was found to be between two "loss" mutational sites. The possibility remains, however, that a protein or protein fragment is produced in some of the non-CRM formers but is not identifiable enzymatically or immunologically as component A or B.

The altered proteins formed by the A and B mutants were examined in enzymatic tests, and it was found that without exception, each was effective (in the presence of the normal second component) in one of the three reactions. It is probably significant that the activity associated with each of the altered proteins is the activity which the *second* (unaltered) component possesses to a slight degree by itself. That is, normal component B catalyzes the indole → tryptophan reaction (but not the other two) to a limited extent alone. With altered A present it catalyzes this reaction at the wild-type (maximal) rate but is still ineffective in the other two reactions. Similarly, the altered B-normal A combination catalyzes the indoleglycerol phosphate→indole reaction at the wild-type rate, while normal A alone can catalyze only this reaction, and at a very low rate. It is perhaps also significant in this regard that in every CRM-forming strain but one, mutant B-2, the altered A or B protein has a normal activity:antigen ratio in the effective reaction. These observations may be taken to indicate that component A is the principal active member in reaction (2) and component B in reaction (1) but until additional information is available on the nature of the interaction between the two proteins, no definitive conclusions can be drawn.

The amounts of A and B protein in the various mutants studied deserves comment; in practically every instance more of these proteins were formed by a mutant than is formed by the wild-type strain. Other studies with *E. coli*[11] have shown that the formation of the enzymes involved in the biosynthesis of tryptophan is subject to repression by excess tryptophan.[12] The medium employed in growing the various mutant strains examined in the present study was supplemented with growth-limiting levels of anthranilic acid, indole, or tryptophan; apparently under these conditions the mutants overproduce the A and B proteins.

The studies performed with revertants, partial revertants, and suppressed mutants demonstrate that restoration of the ability to grow in the absence of tryptophan is correlated with the appearance of an A or B activity which was not present in the mutant. In those strains derived from A mutants it has also been found that the restored activity is stable to acidic conditions which precipitate most *E. coli* proteins, but not component A. Whether the suppressed mutants and partial revertants obtained from CRM-producing mutants form a mixture of altered and active protein or a new species of altered protein is not known, but it is clear that in these strains the restored activity is associated with a considerable excess of immunologically active material. This does not appear to be due to changes in the immunological reactivity of the protein or proteins, since the ability to participate

in one of the three biochemical reactions is usually near normal on a per unit antigen basis. In the suppressed mutants obtained from the A and B non-CRM formers, the restored A or B protein appears normal in enzymatic and immunological properties, but the level of activity restored is unusually low (compared to the unaltered component), indicating that some defect in A or B protein formation persists in these strains.

In several recent studies with *N. crassa* it has been observed that heterocaryons formed between two mutants lacking the same enzymatic activity may produce an active protein.[13–16] Some instances of heterocaryon complementations of this type might result from interactions in two component systems similar to the one described in this paper if the two proteins were required for the catalysis of one reaction, and if each of the mutants contributed the ability to form a different unaltered protein to the heterocaryon. In several instances, however, it has been shown that mixtures of extracts of complementing mutants are enzymatically inactive in the relevant reaction. The possibility exists that in some of these systems the complementing mutants bear defects in nonidentical polypeptide chains which are normally associated in a single protein molecule. In such cases, the normal and altered polypeptide chains contributed to the heterocaryon by the two mutants might be synthesized separately or might be subjected to conditions which favor dissociation and reassociation, thereby permitting the formation of a normal protein. A model for interactions of this type is provided by the recent elegant demonstration by Singer and Itano[18] that the human hemoglobin molecule consists of acid-dissociable nonidentical halves.

Recent discussions based on the Watson-Crick model of DNA have led to the suggestion that nucleotide sequences in DNA segments determine amino acid sequences in specific proteins, and that mutations may result from nucleotide substitutions in DNA.[19] Of considerable interest in this connection are the fine structure and chemical mapping studies of Benzer[20] and Benzer and Freese,[21] and the demonstration by Ingram and co-workers that single amino acid differences distinguish some altered forms of human hemoglobin from the normal form.[22, 23] Considering the results reported here, mutations leading to loss of ability to form the A or B protein could represent "nonsense" mutations,[19] i.e., nucleotide changes in which a new sequence does not specify an amino acid and thus only an incomplete or no polypeptide chain can be formed. Those mutations resulting in the formation of an A or B CRM would then represent instances in which a nucleotide change leads to a different coding sequence and thus a single amino acid substitution in the A or B protein. Whether this interpretation will prove to be correct awaits more detailed genetic and biochemical analysis.

Summary.—The effects of deletions, point mutations, suppressor mutations and reversions on the two components of the TSase of *E. coli* were examined. It was found that deletion mutants lacking the A and B genes also lack the A and B proteins. Point mutants at the A locus and point mutants at the B locus formed either an altered form of the corresponding protein or none at all. In each instance where an altered protein was produced it was enzymatically active in one of the three reactions in which the normal component participates. Full revertants obtained from A and B mutants formed what appears to be normal components while partial revertants and some suppressed mutants produced either a mixture of normal and

abnormal proteins or a new species of active protein. Suppressed mutants derived from non-CRM formers produced relatively little of one of the two components suggesting that some defect in A or B protein formation exists in these strains.

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ON THE LIMIT OF SOLUTIONS OF DIFFERENTIAL-DIFFERENCE EQUATIONS AS THE RETARDATION APPROACHES ZERO

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1. *Introduction.*—In various parts of mathematical physics, we encounter equations of the form

$$\epsilon L_m(u) + L_n(u) = 0 \tag{1.1}$$

where $L_m(u)$ is a differential operator of order m, $L_n(u)$ is a differential operator of

order n, $m > n \geq 1$, and ϵ is a small parameter. Examples of this occur in hydrodynamics where ϵ represents a viscosity, and in the study of the multivibrator (in the form of the Van der Pol equation) where ϵ represents a small inductance. A great deal of effort has been devoted in recent years to the study of the limiting behavior of the solutions of (1.1) as $\epsilon \to 0$.

Equations of this type also arise in parts of the scientific world where time-lags and retardations are present. Given a differential-difference equation of the form

$$\begin{aligned} u'(t) + au(t - \epsilon) &= g(u), \ t > \epsilon, \\ u(t) &= f(t), \ 0 \leq t \leq \epsilon, \end{aligned} \tag{1.2}$$

where $0 < \epsilon \ll 1$, one frequently uses as an approximation an equation of the form

$$u'(t) + au(t) - a\epsilon u'(t) + a\epsilon^2 u''(t)/2 = g(u), \tag{1.3}$$

precisely an equation of the type appearing above.

We shall show that the study of the limiting behavior of the solutions of (1.2) is relatively easy to carry out compared to a similar study for (1.1). A typical result and sketch of proof will be presented below. Complete details, extensions, and generalizations will be presented subsequently.

It is interesting to observe that we have a situation in which the more accurate description yields an easier problem, and the obvious approximation yields a more difficult problem.

2. *A Typical Result.*—Representative of the results that can be obtained is the following.

THEOREM. *Consider the equation*

$$\begin{aligned} u'(t) + au(t - \epsilon) &= g(u), \ t > \epsilon, \\ u(t) &= f(t), \ 0 \leq t \leq \epsilon, \end{aligned} \tag{2.1}$$

where $\epsilon > 0$, *and*

$$\begin{aligned} &(a) \quad g(u) \text{ satisfies a Lipschitz condition for } |u| \leq c_1, \\ &(b) \quad f(t) \text{ is continuous at } t = 0, \\ &(c) \quad |f(0)| = c_2 < c_1. \end{aligned} \tag{2.2}$$

Then, for $0 \leq t \leq t_0$, *where* t_0 *is dependent upon* c_1 *and* $g(u)$,

$$\lim_{\epsilon \to 0} u(t) = v(t), \tag{2.3}$$

where $v(t)$ *is the solution of*

$$v'(t) + av(t) = g(v), \ v(0) = f(0). \tag{2.4}$$

The convergence is uniform for $0 \leq t \leq t_0$.

Similar results can be obtained for systems of equations with any finite number of lags.

3. *Sketch of Proof.*—The proof depends, as might be expected, upon the properties of the solution of the linear equation

$$\begin{aligned} u'(t) + au(t - \epsilon) &= g(t), \ t > \epsilon, \\ u(t) &= f(t), \ 0 \leq t \leq \epsilon, \end{aligned} \tag{3.1}$$

which, in turn, depends upon the properties of the kernel

$$K(t, \epsilon) = \frac{1}{2\pi i}\int_C \frac{e^{st}ds}{s + ae^{-\epsilon s}}. \tag{3.2}$$

Here C represents any line of the form $s = s_0 + it$, $-\infty < t < \infty$, with $s_0 > |a|$. It is easy to show that as $\epsilon \to 0$,

$$K(t, \epsilon) \to K(t) = e^{-at} \tag{3.3}$$

uniformly for $0 \leq t \leq t_0 < \infty$, where t_0 is any fixed finite quantity.

The solution of (1.2), under the assumptions that we have made, can be obtained as the solution of the integral equation

$$u(t) = u_0(t) + \int_\epsilon^t g(u(t_1))K(t - t_1, \epsilon)dt_1, \; t > \epsilon, \tag{3.4}$$

where $u_0(t)$ is the solution of (1) corresponding to $g(u) \equiv 0$.

This we solve by the method of successive approximations. Set

$$\begin{aligned} u_{n+1}(t) &= f(t), \; 0 \leq t \leq \epsilon, \; n \geq 0, \\ u_{n+1}(t) &= u_0(t) + \int_\epsilon^t g(u_n(t_1))K(t - t_1, \epsilon)dt_1, \; t > \epsilon, \; n \geq 0. \end{aligned} \tag{3.5}$$

Under the foregoing assumptions, it is easy to show that $u_n(t) \to u(t)$ uniformly for $0 \leq \epsilon \leq \epsilon_0$, $0 \leq t \leq t_0$, for $n = 0, 1, \ldots$, and that as $\epsilon \to 0$, $u_n(t) - v_n(t) \to 0$, uniformly for $0 \leq t \leq t_0$, where the sequence $\{v_n(t)\}$ is determined by the relations

$$\begin{aligned} v_0(t) &= f(0), \\ v_{n+1}(t) &= v_0(t) + \int_0^t g(v_n(t_1))e^{-a(t - t_1)}\, dt_1, \\ & \qquad t \geqq 0, \; n \geqq 0. \end{aligned} \tag{3.6}$$

Since the same assumptions ensure that $v_n(t) \to v(t)$ uniformly in $0 \leq t \leq t_0$, where $v(t)$ is the solution of

$$v(t) = f(0) + \int_0^t g(v(t_1))e^{-a(t - t_1)}dt_1, \tag{3.7}$$

we have the desired uniform convergence of $u(t)$ to $v(t)$ in $0 \leq t \leq t_0$ as $\epsilon \to 0$.

The same method can be applied to demonstrate the corresponding result for more general differential-difference equations.

THE RICCI PRINCIPAL DIRECTION VECTORS

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1. In a space V_n of n dimensions the Ricci principal direction vectors denoted $\lambda_{h|}{}^i$, as h takes the values 1 to n, are defined by the equations[1]

$$(R_{ij} + \rho_h g_{ij})\, \lambda_{h|}{}^i = 0, \tag{1}$$

where R_{ij} is the Ricci tensor, g_{ij} is the metric tensor, and ρ_h as h takes the values 1 to n, are scalars. Both R_{ij} and g_{ij} are symmetric in their indices i and j.

When in an expression there is an upper and a lower index with the same letter as i in equation (1), the one expression stands for the sum of the n expressions as the repeated index takes the values 1 to n. This convention is used throughout this paper.

The covariant components of the vector $\lambda_{h|}{}^{i}$ are given by

$$\lambda_{h|i} = g_{ij}\lambda_{h|}{}^{j}. \tag{2}$$

The contravariant metric tensor g^{ij} is defined by

$$g^{ij}g_{ik} = \delta_{k}{}^{j}, \tag{3}$$

where $\delta_{k}{}^{j}$ is $+1$ for $j = k$ and is zero for $j \neq k$.

The tensor g^{ij} is used at times to raise an index in the components of a vector or tensor, as g_{ij} is used in equation (2) to lower an index. Since the covariant derivative of g_{ij} and g^{ij} is equal to zero,[2] one of them is used at times to lower or raise an index in the covariant derivative of a tensor. For example

$$\lambda_{h|}{}^{i}{}_{,j|}\cdot\lambda_{k|i} = (g^{im}\lambda_{h|m})_{,j}\lambda_{k|i} = \lambda_{h|m,j}g^{im}\lambda_{k|i} = \lambda_{h|m,j}\lambda_{k|}{}^{m}. \tag{4}$$

From equation (1) written in the form

$$(R_{j}{}^{i} + \rho_{h}\delta_{j}{}^{i})\,\lambda_{h|i} = 0 \tag{5}$$

it follows that the scalars ρ_h are the roots of the determinant equation

$$\left|R_{j}{}^{i} + \rho\delta_{j}{}^{i}\right| = 0. \tag{6}$$

2. For a root ρ_k different from ρ_h there is the equation $(R_{ij} + \rho_k g_{ij})\lambda_{k|}{}^{i} = 0$. When this equation multiplied by $\lambda_{h|}{}^{j}$ and summed for j is subtracted from equation (1) multiplied by $\lambda_{k|}{}^{j}$ and summed for j, the result is within the factor $\rho_h - \rho_k$

$$g_{ij}\lambda_{h|}{}^{i}\lambda_{k|}{}^{j} = \lambda_{h|}{}^{i}\lambda_{|ki} = 0, \tag{7}$$

that is the vectors $\lambda_{k|}{}^{i}$ and $\lambda_{k|}{}^{i}$ at each point of V_n are orthogonal to one another.[3] Hence when for a V_n the roots of equation (6) are simple, the Ricci principal direction vectors form an orthogonal ennuple.

We now consider the determinant in equation (6) written in the form

$$f(\rho) = \rho^{n} + a_{i}\rho^{n-i}, \tag{8}$$

where the a's are functions of the $R_{j}{}^{i}$ and the second term stands for the sum of n terms as i takes the values 1 to n.

The condition that the roots of equation (6) be simple is that the resultant of $f(\rho)$ and its derivative, that is

$$f'(\rho) = n\rho^{n-1} + a_{i}(n - i)\rho^{n-i-1} \tag{9}$$

be different from zero.[4] This resultant is the determinant

$$\begin{vmatrix} a_n, a_{n-1}, \ldots, a_1, 1 & & & \\ 0, a_n, a_{n-1}, \ldots a_1, 1 & & & \\ \ldots\ldots\ldots\ldots\ldots\ldots & & & \\ \ldots\ldots\ldots\ldots\ldots\ldots & & & \\ \ldots\ldots\ldots\ldots\ldots\ldots & & & \\ 0 \quad 0 & & a_n, a_{n-1}, \ldots a_1, 1 & \\ a_{n-1}, \ldots\ldots\ldots (n-2)a_2, (n-1)a_1, n \ldots & & & 0 \\ \ldots\ldots\ldots\ldots\ldots\ldots & & & 0 \\ \ldots\ldots\ldots\ldots\ldots\ldots & & & 0 \\ a_{n-1}\ldots\ldots (n-2)a_2, (n-1)a_1, n & & & \end{vmatrix} \tag{10}$$

where zeros are the terms in spaces otherwise.

As an example we consider the case for a V_3, for which equation (6) is

$$\begin{vmatrix} R^1{}_1 + \rho, & R^1{}_2, & R^1{}_3 \\ R^2{}_1, & R^2{}_2 + \rho, & R^2{}_3 \\ R^3{}_1, & R^3{}_2, & R^3{}_3 + \rho \end{vmatrix} = 0.$$

The expressions for a_i are

$$a_1 = R^1{}_1 + R^2{}_2 + R^3{}_3$$
$$a_2 = R^1{}_1R^2{}_2 + R^2{}_2R^3{}_3 + R^3{}_3R^1{}_1$$
$$a_3 = R^1{}_1R^2{}_2R^3{}_3 + R^1{}_2R^2{}_3R^3{}_1 + R^1{}_3R^3{}_2R^2{}_1 - R^1{}_1R^2{}_3R^3{}_2 - R^2{}_2R^3{}_1R^1{}_3 - R^3{}_3R^1{}_2R^2{}_1,$$

and determinant (10) is

$$\begin{vmatrix} a_3 & a_2 & a_1 & 1 & 0 \\ 0 & a_3 & a_2 & a_1 & 1 \\ a_2 & 2a_1 & 3 & 0 & 0 \\ 0 & a_2 & 2a_1 & 3 & 0 \\ 0 & 0 & a_2 & 2a_1 & 3 \end{vmatrix}.$$

From this example it is seen that for a general V_n the determinant (10) is different from zero, so that the roots of equations (6) are simple. Hence

In a general V_n the Ricci principal direction vectors are an orthogonal ennuple.

3. Scalars γ_{hkl} are defined by $\gamma_{hkl} = \lambda_{h|i}, {}_j\lambda_{k|}{}^i\lambda_{l|}{}^j$. A necessary and sufficient condition that the vectors $\lambda_{h|}{}^i$ of an orthogonal ennuple be normal, that is be the normals to a family of hypersurfaces, is that γ_{hkl} for h, k, l different be equal to zero,[5] namely

$$\lambda_{h|i}, {}_j\lambda_{k|}{}^i\lambda_{l|}{}^j = 0, \qquad (h, k, l \pm). \tag{11}$$

We now determine under what conditions the Ricci principal direction vectors are normal, that is, each set of vectors $\lambda_{h|}{}^i$ is normal to a family of hypersurfaces.

From equations (1) and (7) one has $R_{ij}, \lambda_{h|}{}^i\lambda_{k|}{}^j = 0$, of which the covariant derivative with respect to x^t is $R_{ij}, {}_t\lambda_{h|}{}^i\lambda_{k|}{}^j + R_{ij}\lambda_{h|}{}^i, {}_t\lambda_{k|}{}^j + R_{ij}\lambda_{h|}{}^i\lambda_{k|}{}^j, {}_t = 0$.
By means of equations of the form [1] this equation may be given the form

$$R_{ij}, {}_t\lambda_{h|}{}^i\lambda_{k|}{}^j - \rho_k\lambda_{k|t}\lambda_{h|}{}^i, {}_t - \rho_k\lambda_{h|j}\lambda_{k|}{}^j, {}_t = 0. \tag{12}$$

From (7) we have $\lambda_{h|j}\lambda_{k|}{}^j = 0$, of which the covariant derivative with respect to x^t is $\lambda_{h|j}, {}_t\lambda_{k|}{}^j + \lambda_{h|j}\lambda_{k|}, {}^j{}_t = 0$.

Since $\lambda_{h|j,\ i} = (\lambda_{h|}{}^{t}g_{tj})_{,\ i} = g_{tj}\lambda_{h|}{}^{t}{}_{,\ i}$, the above equation may be given the form $\lambda_{h|}{}^{t}{}_{,\ i}\lambda_{k|t} + \lambda_{h|j}\lambda_{k|,}{}^{j}{}_{i} = 0$.

By means of this result equation (12) becomes

$$R_{ij,\ i}\lambda_{h|}{}^{i}\lambda_{k|}{}^{j} + (\rho_h - \rho_k)\lambda_{k|}{}_{i}\lambda_{h|}{}^{t}{}_{,\ i} = 0,$$

which may be given the form

$$R_{ij,\ i}\lambda_{h|}{}^{i}\lambda_{k|}{}^{j} + (\rho_h - \rho_k)\lambda_{k|}{}^{i}\lambda_{h|t,\ i} = 0.$$

We multiply this equation by $\lambda_{m|}{}^{t}$, sum for l and get

$$R_{ij,\ i}\lambda_{h|}{}^{i}\lambda_{k|}{}^{j}\lambda_{m|}{}^{t} + (\rho_h - \rho_k)\lambda_{k|}{}^{i}\lambda_{m|}{}^{t}\lambda_{h|t,\ i} = 0. \tag{13}$$

We require that V_n is such that

$$R_{ij,\ i} = {}^{1}/_{2}R_{,i}g_{jt} + {}^{1}/_{2}R_{,j}g_{it}, \tag{14}$$

where R is the Ricci scalar, that is $R = g^{ij}R_{ij}$. When equation (14) is multiplied by g^{ij} and contracted for i and j, the result is satisfied identically.

For $R_{ij,\ i}$ as given in (14) and h, k, l different, the first term of equation (13) is equal to zero in accordance with equations (7) and since the ρ's are different one has $\lambda_{h|t,\ i}\lambda_{k|}{}^{i}\lambda_{m|}{}^{t} = 0$, $(h, k, m \pm)$. Hence the vectors are normal. Since similar results hold for all the principal director vectors, we have

In a V_n for which equation (14) holds the Ricci principal direction vectors are normal.

[1] Eisenhart, L. P., *Riemannian Geometry* (Princeton University Press, 1926, 1944), equation (34.4), 114.

[2] *Ibid.*, equation (21.8), 28.

[3] *Ibid.*, equation (13.5), 38.

[4] Walker, R. S., *Algebraic Curves* (Princeton University Press, 1950), 24, 25.

[5] Eisenhart, L. P., *ibid.*, equation (30.1), 97, and equation (36.1), 117.

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*AN EXTENSION OF CERTAIN RESULTS IN FUNCTION THEORY TO A CLASS OF SURFACES**

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Communicated by Lars V. Ahlfors, May 1, 1959

The object of this paper is to show that a large number of results in function theory can be carried over from plane regions to a certain well-defined class of differential-geometric surfaces. The definition of this class is not constructive, so that the difficult part is to prove that a given surface belongs to the class. However, we do show that the class includes a large class of minimal surfaces, which in turn includes all simply-connected minimal surfaces representable in the form $z = f(x, y)$, and the results obtained are of interest even for this restricted class of surfaces. An example of the type of theorem obtained for this case is the following analogue of the Koebe $\frac{1}{4}$-Theorem.

Let S be a simply-connected minimal surface of the form $z = f(x, y)$ and let p be a point of S whose geodesic distance to the boundary of S is at least d. If ds is the ele-

ment of arc length on S, and if S is mapped one-to-one conformally onto a region R in the complex w-plane with the normalization $|dw|/ds = 1$ at p, then every boundary point of R is at a distance at least d/8 from the image of p.

Definition: Let S be a surface with a Riemannian metric ds^2. We shall say that S belongs to the class $\mathfrak{S}(m)$ if there exists a mapping of S into the complex z-plane which is conformal with respect to the metric on S and which satisfies

$$1 \leq \frac{|dz|}{ds} \leq m \tag{1}$$

throughout S.

The mapping is not required to be globally one-to-one, but the image of S will at any rate be an unbranched Riemann surface lying over the z-plane.

LEMMA 1. *Let $S \in \mathfrak{S}(m)$ and let p be a point of S whose distance to the boundary of S is at least d. Then there exists a one-to-one conformal map of the disk $|z| < d$ into S such that the point $z = 0$ maps into p, and*

$$\frac{1}{m} \leq \left[\frac{ds}{|dz|}\right]_{z=0} \leq 1.$$

Proof: By the definition of $\mathfrak{S}(m)$, S may be mapped conformally onto an unbranched Riemann surface R over the z-plane, and we may assume that the image q of p lies over the origin. Let D be the largest open disk about q lying in R. Then D projects onto a disk $|z| < r \leq \infty$. We wish to show that $r \geq d$. If $r = \infty$, there is nothing more to prove. If not, there must exist a point z_1 with $|z_1| = r$ such that the relative boundary of D contains no point over z_1. Let L be the segment in D projecting onto the line segment from 0 to z_1. Then L corresponds to an arc C in S which goes from p to the boundary of S. Hence, using (1), we have

$$d \leq \int_C ds \leq \int_L |dz| = r,$$

which proves the lemma.

COROLLARY. *A complete simply-connected surface $S \in \mathfrak{S}(m)$ is conformally equivalent to the plane.*

Proof: The image Riemann surface R over the z-plane has no algebraic branch points and no boundary points at finite distance and must therefore coincide with the z-plane.

THEOREM 1. *Let $S \in \mathfrak{S}(m)$ and let p be a point of S whose distance to the boundary of S is at least d. Let S be mapped one-to-one conformally into the w-plane with the normalization $|dw|/ds = 1$ at p. Then the image region includes a disk of radius $d/4m$ about the image of p.*

Proof: By Lemma 1, we get a one-to-one conformal map $w = f(z)$ of the disk $|z| < d$ into the w-plane such that $|f'(0)| = [|dw|/ds]_p \cdot [ds/|dz|]_0 \geq 1/m$. By the Koebe $\frac{1}{4}$-Theorem the image region must contain a disk about $f(0)$ of radius $d/4m$.

We note that any plane region, considered as a surface S in the euclidean metric, belongs to $\mathfrak{S}(1)$ by virtue of the identity map. Theorem 1 therefore reduces in the case of plane regions to the classical Koebe Theorem.

Definition: The class $\mathfrak{M}(\alpha)$ is defined to be the class of all simply-connected minimal surfaces S having the property that all normals to S make an angle of at least $\alpha > 0$ with some fixed direction.

We note that a minimal surface of the form $z = f(x, y)$ belongs to the class $\mathfrak{M}(\pi/2)$.

THEOREM 2. *$\mathfrak{M}(\alpha) \subset \mathfrak{S}(m)$ for $m = \csc^2 \frac{\alpha}{2}$.*

Proof: Let $S \in \mathfrak{M}(\alpha)$. Since S is a minimal surface, the map of S by parallel normals into the unit sphere $\sum$ is conformal, and if $d\sigma$ is the line element on $\sum$ we have $d\sigma^2/ds^2 = |K|$, where K is the Gauss curvature of S. We project $\sum$ stereographically from the point corresponding to the distinguished direction in space onto the complex w-plane. Since the image of S in $\sum$ is bounded away from the distinguished direction by at least α, the image of S in the w-plane will lie in the disk $|w| < R$, where $R = \cot \frac{\alpha}{2}$. We may write the line element $d\sigma$ on $\sum$ as $d\sigma = \lambda |dw|$ where $\lambda = 2/(1 + |w|^2)$. Let $h(p)$ be the function on S defined to have at each point the value of λ at the corresponding point in the w-plane. Then

$$\frac{2}{1 + R^2} < h(p) \leq 2 \tag{2}$$

on S. We shall show that there exists a locally conformal map of S into the complex ζ-plane satisfying

$$\left[\frac{|d\zeta|}{ds}\right]_p = h(p) \tag{3}$$

throughout S. We give the proof in the case that K is nowhere zero on S. This restriction can be lifted by a slight additional argument[1] at points where $K = 0$.

For $K \neq 0$ the map of S into $|w| < R$ is locally one-to-one, and the image of S will be a simply-connected unbranched Riemann surface W over the w-plane. In the neighborhood of each point of S we may write $ds = \rho |dw|$, where

$$\rho = \frac{ds}{|dw|} = \frac{ds}{d\sigma} \cdot \frac{d\sigma}{|dw|} = \lambda / \sqrt{|K|}\,. \tag{4}$$

The formula for the Gauss curvature in isothermal coordinates is $K = -(\Delta \log \rho)/\rho^2$, where Δ is the Laplacian with respect to w. This gives

$$\Delta \log \rho = -K\rho^2. \tag{5}$$

The same formula applied to $\sum$ yields $-(\Delta \log \lambda)/\lambda^2 = 1$, or

$$\Delta \log \lambda = -\lambda^2 = -\rho^2 |K| = K\rho^2 \tag{6}$$

where we have used (4) and the fact that $K \leq 0$ since S is a minimal surface.

Combining (5) and (6) we see that the function $\varphi = \log \rho\lambda$ is a harmonic function on W. By the simple-connectivity of W we can find a harmonic conjugate ψ. We then define

$$\zeta = \int e^{\varphi + i\psi}\, dw \tag{7}$$

where the integral is taken along paths on W from a fixed point to a variable point. Then $|d\zeta/dw| = e^{\varphi} = \rho\lambda$, and since $ds/|dw| = \rho$, we obtain $|d\zeta|/ds = \lambda$, which proves (3).

Finally, if we let $z = \zeta(1 + R^2)/2$ and use (2) we obtain (1) with $m = 1 + R^2$. This proves the theorem.

In particular, we see that every simply-connected minimal surface of the form $z = f(x, y)$ is contained in $\mathcal{S}(2)$, which combined with Theorem 1 gives the result stated at the beginning of the paper.

We note further, combining Theorem 2 with the corollary to Lemma 1, that every complete surface S in $\mathfrak{M}(\alpha)$ is conformally equivalent to the plane. By Liouville's Theorem, the map of S into $|w| < R$ must be a constant. Thus S has a single normal direction, and we obtain the following result, which was conjectured by L. Nirenberg[1]:

Every complete surface $S \in \mathfrak{M}(\alpha)$ must be a plane.

This generalizes the theorem of Bernstein that a minimal surface of the form $z = f(x, y)$ over the whole x,y-plane must itself be a plane.

It is clear that by using Lemma 1, one can derive for surfaces in $\mathcal{S}(m)$ analogues of a wide class of distortion theorems in the plane, including, for example, Schwarz' lemma, Bloch's Theorem[5] and Schottky's Theorem. By applying these results to surfaces in $\mathfrak{M}(\alpha)$ we obtain further geometrical properties of minimal surfaces.

The following generalization of Schwarz' lemma is stated for mappings of one surface into another.

THEOREM 3. *Let $S_1 \in \mathcal{S}(m)$ and $S_2 \in \mathcal{S}(n)$. Let p be a point of S_1 whose distance to the boundary of S_1 is d_1 and let f be an analytic map of S_1 into S_2. If every point of $f(S_1)$ is a distance less than d_2 from $f(p)$, then*

$$\left[\frac{ds_2}{ds_1}\right]_p \leq mn\,\frac{d_2}{d_1}$$

where ds_i is the element of arc length on S_i, $i = 1,2$.

The proof is again a direct application of Lemma 1.

Applying Theorem 3 to the particular map, constructed in the proof of Theorem 2, which takes a surface S in $\mathfrak{M}(\alpha)$ into the disk $|w| < R$, we obtain the following analogue for the class $\mathfrak{M}(\alpha)$ of an inequality due to Heinz[2] in the special case of minimal surfaces of the form $z = f(x, y)$:

If $S \in \mathfrak{M}(\alpha)$, and p is a point of S whose distance to the boundary is at least d, then the Gauss curvature K at p satisfies

$$|K| \leq \frac{1}{d^2}\left(2\cot\frac{\alpha}{2}\csc^2\frac{\alpha}{2}\right)^2.$$

A stronger inequality, due to Hopf,[3] can also be extended easily to the class $\mathfrak{M}(\alpha)$.[4]

* This work was supported in part by the Office of Ordnance Research, U.S. Army.

[1] Osserman, R., "Proof of a conjecture of Nirenberg." *Comm. Pure Appl. Math.* (to appear).

[2] Heinz, E., "Uber die Lösungen der Minimalflächengleichung," *Nachr. Akad. Wiss. Gottinge Math.-Phys. Kl.*, 51–56 (1952).

[3] Hopf, E., "On an inequality for minimal surfaces $z = z(x, y)$." *J. Rational Mech. Anal.*, **2**, 519–522 (1953).

[4] Osserman, R., "An analogue of the Heinz-Hopf inequality." *J. Math. Mech.* (to appear).

[5] (*Added in proof*) A form of Bloch's Theorem had been obtained previously by R. Finn for a function of the form $z + i\zeta$ associated with a minimal surface $z = f(x, y)$. Subsequent conversations have revealed that Finn's proof uses some of the ideas presented here. The connection is

clear if one observes that the z-coordinate on a minimal surface is a harmonic function on the surface, so that one may complete it to an analytic function $z + i\zeta$, and apply the above methods.

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*THE PROPAGATION OF MULTIPLE VIRUSES IN CHICK KIDNEY CULTURES**

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Introduction.—Efforts have been directed toward developing another tissue culture system in which viruses like influenza, adenovirus, measles, hemadsorption type I and II, and poliovirus can be propagated. The ability to grow all of these viruses in the same type of cells would permit a comparison of many of their biologic properties. Buthala and Mathews[1] have investigated the susceptibility of cultures of embryonic chick kidney cells to different animal viruses and Wright and Sagik[2] followed up this work with the study of plaque formation by these viruses in the same culture system. We found that the kidney of a fully developed chick could be excised with greater ease than that of an embryonic chick, that the yield of cells was considerably higher, and that a number of laboratory passaged viruses could be propagated in the monkeys obtained. The system has also been found suitable for primary isolation of viruses.

TABLE 1

"VIRAL SPECTRUM" OF THE CHICK KIDNEY SYSTEM

Virus	Passage History†	Virus Titer* TCID$_{50}$ per ml. 0 days	3 days	6 days
Influenza				
A (A/AA/1/58)	TW-CK$_5$	1.5	4.5	5.3
B (Lee)	E$_4$F$_3$M48E$_{50}$-CK$_5$	2.0	3.8	4.5
C (JJ)	AE$_{43}$	1.8	0	0
Adenovirus	HeLa cells$_{20}$			
Type 4	CK$_5$	2.0	3.0	4.3
Hemadsorption				
Type I	MK-CK$_5$	1.8	4.5	5.3
Type II	MK-CK$_5$	2.3	4.7	5.0
Measles	Chick embryo cells$_{13}$-CK$_5$	1.3	2.8	4.0
Polio	MK-CK$_5$	1.0	2.7	3.8
Type I	Stool specimen-CK$_5$	1.3	3.3	4.3

* All titers are expressed as the negative log of the dilution which will infect 50 per cent of inoculated chick kidney cultures tubes.

† CK$_5$—The fifth passage in chick kidney cells; TW—throat washing; AE—Embryonate eggs (Amniotic route); E—Embyronate eggs (Allantoic route); M—Mouse; MK—Monkey kidney.

The data summarized in Table 1 show that the viruses studied grew readily in this type of culture. A line of virus which has been cultivated in a different tissue system requires a period of adaptation before it reaches maximal growth in chick cells, while a line directly isolated in chick cells multiplies to a relatively high titer promptly. The infectivity titers of some of the agents like influenza and polio were lower than expected when measured after five transfers. However, it can be

anticipated that with further passages and/or modification in cultural conditions, better titers may be obtained.

The advantages of the chick kidney system are: it avoids the use of continuous cell cultures; it is cheaper than monkey kidney cultures; the tissue is readily available in large amounts; and to date no "wild viruses" have been encountered.

Procedure.—In all instances, kidneys from 4 to 5 day old chicks were used. The cells were prepared by a modification of the procedure of Youngner.[3] The growth medium consisted of lactalbumin hydrolysate (90 per cent of 0.5 per cent solution in Hank's balanced salt) and 10 per cent calf serum. The suspension of cells obtained was diluted so that each ml contained approximately 3×10^5 cells. Various concentrations of cells were used to seed different sizes of specially washed glass culture containers. The growth medium was removed at the sixth day and a new medium composed of Eagle's basal solution[4] and 5 per cent calf serum was substituted. This procedure was found most suitable for obtaining a uniform monolayer of cells. The cultures were used at the eighth day. The maintenance medium used to propagate the different viruses consisted of double strength Eagle's solution and 2 per cent calf serum or, in the case of influenza virus, double strength Eagle's basal media without serum.

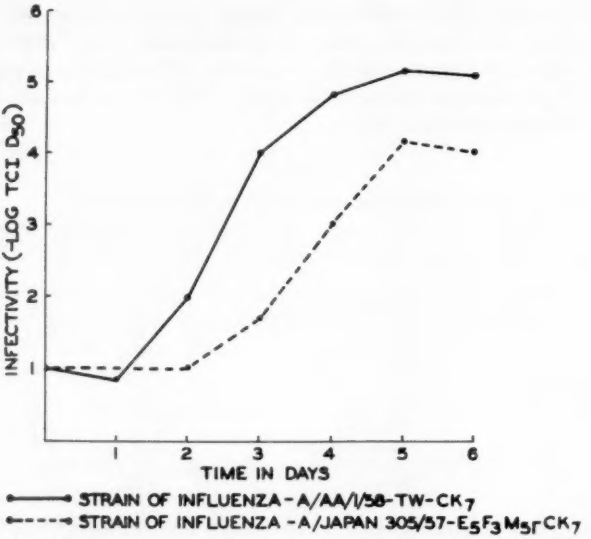

FIG. 1.—Growth curves of two lines of influenza A virus in chick kidney cultures.

Influenza Virus.—Both types A and B influenza virus produced cellular destruction during growth in the chick kidney cells. The infectivity titer of a line directly isolated from a throat washing was higher than titers of lines adapted to eggs or mice. The growth curves of two different lines of type A virus are shown in Figure 1. The data were obtained from cultures inoculated with about 50 $TCID_{50}$

of virus per cell. After three hours of incubation at 37°C, the cells were washed free of residual inoculum and fresh culture fluid was added. Samples were taken at daily intervals. It will be noted in the growth curve of the line isolated in tissue culture, that there is a lag period of about one day followed by a release period of at least three days. By the fourth day the maximal virus yield had been obtained since no further significant increase in titer was measured. Results obtained using the egg line of influenza A virus show a pattern of growth with a longer lag phase and the final virus yield was smaller.

Plaque Formation.—The plaque assay method of Dulbecco and Vogt[5] was modified for the use of chick kidney cell cultures. The cultures were prepared as described above. The cells were washed three times with Hank's balanced salt solution prior to the addition of virus. The strain of virus used was the Asian strain (A/AA/1/58) influenza A virus. The composition of the final agar overlay consisted of double strength Eagle's basal solution, 0.15 per cent Difco's Noble Agar, plus 100 units of penicillin, mycostatin, and 100 micrograms of streptomycin; finally solution was adjusted to pH 7.3 with 2.8 per cent $NaHCO_3$. The period of adsorption of the virus inoculum was four hours at 36°C before addition of the agar overlay. Each day, from the second to the eighth day following infection, neutral red (3×10^{-4}) was added and the number of plaques counted. It was found that influenza virus will produce plaques with an average size of 4 mm. The production of countable plaques started at the third day and progressed until the fifth day when the maximum number of plaques was reached. There was a nonspecific cellular degeneration after the sixth day of infection and it was found difficult to count the number of plaques with accuracy.

Measles Virus.—The Edmonston strain of measles virus will multiply in this culture system. Figure 2 shows a growth curve of the virus in chick kidney cells.

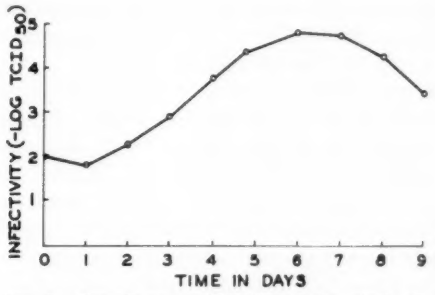

FIG. 2.—Growth curve of the Edmonston strain of measles virus in chick kidney cultures.

The virus did not require a period of adaptation. The maximum titer of about $10^{-4.3}$ is reached after approximately six days of incubation at 37°C. Cytopathogenic changes start at the third day and continue until the entire cell sheet is involved. The first increase in infectivity is evident on the second day of infection and progresses slowly until the sixth day. If the incubation of the infected culture is extended beyond the seventh day a marked decrease in infectivity titer occurs.

Adenovirus.—The chick kidney cells will support the growth of adenovirus, type 4. The rate of growth of the virus is slow. Maximum titer of $10^{-4.0}$ is reached on the seventh day. This is higher than ordinarily obtained in other systems. The progressive increase in titer from first to sixth passages is presented in Table 2.

TABLE 2

GROWTH PATTERN OF ADENOVIRUS TYPE 4 (RI-67*) ON SERIAL PASSAGES IN CHICK KIDNEY CULTURE

Passage Number	Infectivity Titer† ($TCD_{50/ml\ log}$)	Cytopathology
1	2.0	0
2	2.0	0
3	2.7	±
4	3.0	+
5	4.0	+
6	4.0	+

* The RI-67 virus was obtained from Dr. Hilleman and was passaged four times in HeLa cells in this laboratory.

† The values represent the reciprocal logarithm of dilution of the infectivity titer per ml at the 7th day after infection.

It will be noted that there was no increase in titer and there was no cytopathic effect during the first two passages. At the third passage a rise in titer followed by cytopathogenic changes was observed. The cytopathogenic changes were at first localized at the periphery of the monolayer culture. By the eleventh passage the changes occurred throughout the cell sheet. The amount of virus present was determined by disrupting the infected cells by quick freezing and thawing five times in an ethanol-dry ice mixture. Cultivation of other types of adenovirus has not yet been attempted.

Hemadsorption Viruses.—The growth pattern of the hemadsorption type I and II viruses is analogous to adenovirus type 4. Three serial passages in chick cells were required for adaptation of both agents as indicated by an increase in infectivity titer. The highest titer is reached on the sixth day after infection. Definite cellular alterations were evident on the third day and the hemadsorption reaction became positive approximately twelve hours prior to the appearance of cytopathogenic changes and the release of virus into the medium.

Poliovirus.—The Mahoney strain of poliovirus type I was found to multiply in chick cells. The infection of these cells by that virus is not efficient and the highest titer obtained at the eighth passage after five days of incubation at 37°C was $10^{3.7}$ $TCID_{50}/_{ml}$. The poliovirus persisted in the chick kidney cells with no concomitant increase for four passages. On the fifth passage an increase in infectivity titer and cytopathogenic changes occurred. The infectivity titer of poliovirus in chick kidney cells is lower than the corresponding titer in monkey kidney culture. The release of virus from the cells is extended over a 5-day period and the cytopathogenic effect is always confined to portions of the cell sheet. This is probably a reflection of the heterogeneity of the cell population with diverse cell types unable to support the growth of poliovirus. Attempts to obtain plaques with poliovirus in chick cells have been unsuccessful.

Isolation of type I poliovirus from stools was also successful. The infectivity titer and the rate of growth were higher and faster respectively for the isolate than for the adapted strain. The conditions for growth presented by the chick kidney cells are obviously different from those in monkey kidney cells; nevertheless, they were quite suitable for isolation of virus and for maintenance of poliovirus. The monkey cell strain was less well maintained.

Cytopathology.—In cultures similar to those in which the development of virus was followed, stained preparations of the cells were made at intervals. In this manner, it has been possible to identify infected cells, to describe the sequence of the cytological changes and to estimate the degree of involvement of the culture. Cultures infected with influenza virus show an increase in granularity, with rounding, enlargement of cells followed by pyknosis of the nucleus, ending in cellular fragmentation and death. The cellular changes as a result of infection with adenovirus type 4 are typified by rounding, and fusion of cells into clusters with increased granularity. The cytopathogenic changes associated with measles are similar to those described by Katz *et al.*[6] in chick embryo cultures. The involved cells exhibited a fusiform shape together with syncytia or multinuclear giant cells. Hemadsorption type I and II produce definite cytopathogenic changes characterized by focal areas of involvement with normal appearance of surrounding cells; the cytopathic effect of these two viruses are indistinguishable. The changes in cells infected by poliovirus consist of localized areas in which there is rounding, loss in cytoplasmic volume, increase in numbers of refractile cells, and pyknosis of the nucleus.

In summary, a chick kidney culture system has been developed in which a wide spectrum of viruses was propagated successfully. Factors have been described which influence the propagation of these agents. The growth of the viruses was always accompanied by cytopathic changes in the chick kidney cultures.

* This investigation was conducted under the auspices of the Commission on Influenza, Armed Forces Epidemiological Board, Office of the Surgeon General, U.S. Army, Washington, D.C.

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[2] Wright, B. S., and B. P. Sagik, *Virology*, **5**, 573 (1958).
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[5] Dulbecco, R., and M. Vogt, *J. Exp. Med.*, **97**, 167 (1954).
[6] Katz, S. L., M. V. Milovanovic, and J. F. Enders, *Proc. Soc. Exp. Biol. and Med.*, **97**, 23 (1958)

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*THE RELATIVE HOMOGENEITY OF MICROBIAL DNA**

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Density-gradient centrifugation has revealed that the population of DNA molecules from *E. coli* is relatively homogeneous with respect to buoyant density in a solution of cesium chloride.[1] Because of the notably small apparent atomic volume of nitrogen in aqueous solutions of organic compounds[2] and because the guanine-cytosine base pair is more rich in nitrogen than is the adenine-thymine pair, it was considered that the density homogeneity among *E. coli* DNA molecules might reflect a high degree of homogeneity with respect to base composition. To investigate this possibility, an examination has been made of the relationship between buoyant density in cesium chloride solution and base composition of DNA from various sources.

We have measured the buoyant density of nine different bacterial DNA's ranging from 0.3 to 0.7 in mean molar fraction of guanine plus cytosine as determined by chromatographic analysis in one laboratory.[3] The DNA from each bacterial species formed a single sharp band in the cesium chloride density gradient. The mean buoyant density of each banded bacterial DNA was determined with respect to the band formed by an aliquot of $C^{13}N^{15}$ *E. coli* DNA which served as a convenient density reference. Typical results are shown in Figure 1, which shows the bands

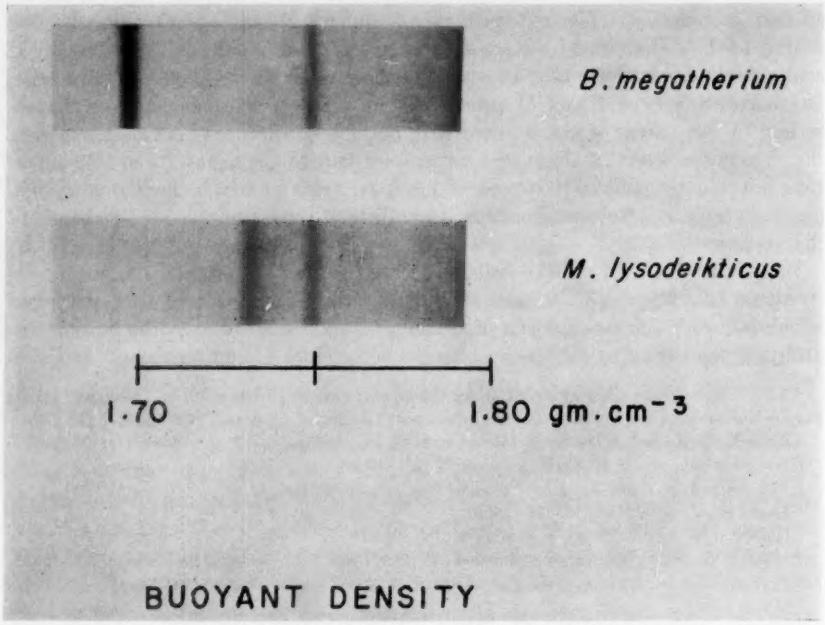

FIG. 1.—Ultraviolet absorption photographs showing bands of DNA from *B. megatherium* and *M. lysodeikticus* with reference bands of $C^{13}N^{15}$ *E. coli* DNA in 57.5 weight percent CsCl solution after 24 hours of centrifugation at 44,770 rpm. All bacteria were grown in broth at 37° C. to a titer of 2×10^8 and sedimented in the cold for ten minutes at 1800 × g. The pellet was resuspended in 0.4 ml. of a solution 0.3 *M* in NaCl, 0.01 *M* in sodium citrate, 0.015 *M* in versene, and 0.01 *M* in tris(hydroxymethyl)aminomethane buffered at pH 6.1. Bacteria were lysed with 0.1 ml. 10 percent sodium dodecyl sulfate or 5 percent sodium desoxycholate, and in some cases were exposed to 100 micrograms/ml lysozyme for 30 seconds before detergent was added. Conditions of ultracentrifugation of the lysates have been described previously.[1]

formed by DNA from *B. megatherium* and from *M. lysodeikticus* and the accompanying reference bands of $C^{13}N^{15}$ *E. coli* DNA. As may be seen from Figure 2, a linear relationship[4] given by the expression

$$\rho_{25°} = 1.658 + 0.100\ \mathrm{GC\ gm.\ cm^{-3}}.$$

is found between the mean buoyant density ρ at 25°C and the mean guanine-cystosine content GC defined as the molar ratio $\frac{G+C}{G+C+A+T}$. The observed value of $\frac{d\rho}{dGC}$ is considerably larger than the value 0.038 estimated from apparent atomic volumes in aqueous solution.[2]

Equation (1) was obtained from determinations of the mean composition and mean buoyant density of DNA from several species of bacteria but we may now apply it to the population of DNA molecules from a single species. In particular,

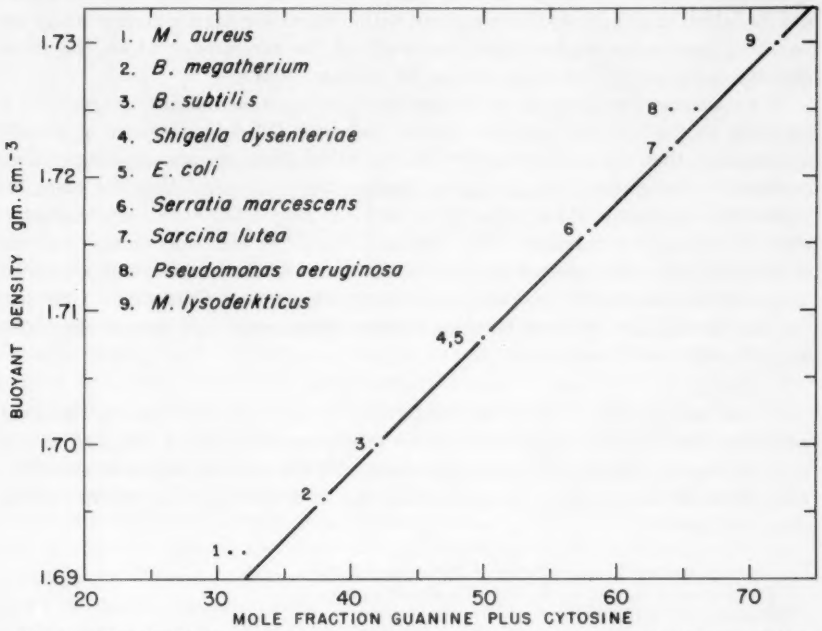

FIG. 2.—The density-composition relationship for DNA. Mean buoyant density at 25°C. ($P_{25}°$) is plotted against mean molar fraction of guanine plus cytosine (GC) for the DNA from nine bacterial species. Where GC is variable among the studied strains of a given species, the range of variation is indicated by a pair of points. The base composition given for *Shigella dysenteriae* is that of *Shigella paradysenteriae*.

we shall compute for each species an upper bound on the standard deviation σ_{GC} of the distribution of the guanine-cytosine base pair over the population of DNA molecules. From equation (1) the upper bound on σ_{GC} is given as

$$\sigma_{GC\ \mathrm{max.}} = 10.0\sigma_\rho$$

where σ_ρ is the standard deviation of the DNA distribution in the cesium chloride density gradient. This computation requires for its validity that no DNA band be seriously narrowed by intermolecular aggregation and that each band comprise a representative sample of the total DNA of the corresponding bacterial species. Aggregation is unlikely in view of the absence of aggregation in a cesium chloride density gradient between molecules of *E. coli* DNA differing only in their content of the heavy isotope N^{15}.[1] It is very likely that the banded DNA is representative of the total DNA because of the close agreement between the mean GC content determined chromatographically and that calculated from buoyant density by means of equation (1). It should be pointed out that the actual value of σ_{GC} may lie considerably below the calculated upper bound because thermal motion of the DNA molecules contributes significantly to the bandwidth.

The DNA from each bacterial species investigated forms a band in the density gradient with σ_ρ in no case greater than 0.003 gm. cm^{-3}. The corresponding upper bound on the standard deviation σ_{GC} of the molecular content of guanine plus cytosine is therefore in no case greater than 0.03.[5] It is remarkable that the standard deviation of guanine-cytosine content within the molecular population of any one bacterial species covers less than one tenth of the range over which the mean guanine-cytosine content varies among the various species.

If we assume for purposes of discussion that much hereditary information is common to the various bacterial species and that DNA is a carrier of genetic information, then our finding that few, if any, DNA molecules possess compositions common to the various species argues against the conception that the complete nucleotide sequence could in principle be deduced from other hereditary characteristics according to a universal code. Instead, it may be that only certain features of the nucleotide sequence are genetically significant so that extensive modification of nucleotide composition need not result in any other genetic alteration. Or it may be that the detailed relation between nucleotide sequence and genetic specificity is itself a species characteristic.

As this investigation was being completed, we learned that Sueoka, Marmur, and Doty have independently arrived at a relation between DNA composition and buoyant density which is in good agreement with the relation reported here. We wish to thank these authors for communicating their findings to us before publication.

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‡ Contribution No. 2460.

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[4] The mean buoyant densities of calf thymus, salmon sperm, and human leukocyte DNA all lie close to the values calculated from equation (1). However, the density distribution of the DNA from each of these vertebrate sources exhibits marked skewness toward higher density (M. Meselson, Thesis, California Institute of Technology, 1957). This skewness might reflect an uneven distribution of GC among the molecules of DNA or may be due to an uneven distribution of the rare base 5-methyl cytosine which occurs in small amounts in the DNA from these three sources.

The density composition relationship (1) is not valid for every DNA which has been examined. Density values considerably higher than those calculated from (1) are observed for heat denatured DNA from *E. coli*, calf thymus, and salmon sperm,[1] for T2 and T4 phage DNA in which cytosine is replaced by 5-hydroxymethyl cytosine or glucosylated 5-hydroxymethyl cytosine, and for DNA from the phage ϕX 174, found to have an unusual structure and composition [Sinsheimer, R. L., *J. Molec. Biol.* (in press)]. These exceptions indicate that the density composition relationship is valid only for a particular class of DNA, possibly for native two-stranded DNA containing no unusual bases.

[5] It might be of interest to compare our estimate of the upper bound on the standard deviation σ_{GC} with the value to be expected for a population of DNA molecules each containing N base pairs assembled at random from a collection of base pairs in which guanine-cytosine occurs with fixed probability one half.

The standard deviation σ_{GC} for this case is just $\frac{1}{2\sqrt{N}}$. Taking 10^4 as a reasonable value of N

for bacterial DNA[1] there results the value $\sigma_{GG} = 5 \times 10^{-3}$, well below the upper limit set by the density gradient experiments. Accordingly, such statistical heterogeneity is not ruled out.

STRAIN SPECIFICITY AND PRODUCTION OF ANTIBIOTIC SUBSTANCES. X. CHARACTERIZATION AND CLASSIFICATION OF SPECIES WITHIN THE STREPTOMYCES GRISEUS GROUP

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In 1948, a detailed description of the streptomycin-producing organism was published[13] under the name *Streptomyces griseus* (Krainsky) Waksman and Henrici. In the eleven years that have since passed, there has accumulated a very extensive literature dealing with this organism and a number of related forms. Numerous varieties have been described, and a certain degree of confusion has arisen. It was thereby established that we are dealing here not with a single species, but with a group of closely related forms. Because of the great economic importance of this group, its re-evaluation and reinterpretation is justified at this time.

Nomenclature of the Streptomycin-Producing Organism.—In 1916, Waksman and Curtis[11] isolated from different soils cultures of an organism which they believed to be similar to a culture previously isolated and described as *Actinomyces griseus* by Krainsky.[5] Waksman[10] soon recognized, however, that the two organisms were slightly different. Since no type culture of Krainsky's organism has ever been available for comparison, all the descriptions subsequently published in the various editions of Bergey's *Manual of Determinative Bacteriology* were based upon that of Waksman and Curtis,[11] later modified by Waksman,[10] and by Waksman *et al.*[13] When Waksman and Henrici[12] proposed, in 1943, to create a new genus *Streptomyces*, this organism was named *Streptomyces griseus*. The strain to which this name was applied carries our laboratory number 3463. It was the first streptomycin-producing culture and has been designated as the type culture (by original designation) of the new species *Streptomyces griseus* Waksman and Henrici.

The original proposal to create a new generic name *Streptomyces* was made by Waksman and Henrici in a manuscript submitted to the *Journal of Bacteriology* in April, 1943, and to Dr. R. S. Breed in May, 1943, for publication in the sixth edition of Bergey's *Manual*. This name first appeared in the *Journal of Bacteriology* in October, 1943; unfortunately, the sixth edition of Bergey's *Manual*, which contained a detailed description of *Streptomyces griseus* was not published until 1948.

Krassilnikov[6] also recognized a difference between the culture isolated by Krainsky and that of Waksman and Curtis. The organisms exhibiting spiral formation were regarded as characteristic of Krainsky's culture; the nonspiral forming strains characteristic of the streptomycin-producing *S. griseus* were regarded as a subspecies of *A. globisporus*, and designated as *A. globisporus streptomycini*, later changed to *A. streptomycini*.[7] Krainsky's culture, however, has never been seen by

Krassilnikov, and because Krainsky did not describe the morphology of the aerial mycelium it will remain unknown forever whether his culture formed spirals or not. Furthermore, the color of the aerial mycelium and the pigment of Krainsky's *A. griseus*, as described by him and as can be seen in the color photograph of his publication, resembles the streptomycin-producing culture more than the *A. griseus* of Krassilnikov. Since the Waksman and Henrici characterization of the streptomycin-producing organism, based upon the description by Waksman and Curtis, is now universally accepted, there is hardly any justification for changing the name.

Harada *et al.*[3] concluded as well that Krassilnikov was wrong in placing the streptomycin-producing organism with *A. globisporus* for the simple reason that the former produces oval to cylindrical spores, whereas the latter produces round spores, as its name indicates.

Baldacci and Comaschi[1] suggested that Krainsky's original culture of *A. griseus* appears to have been a strain of *S. viridis* Lombardo Pellegrino. They state:

"The comparison between Krainsky's and Waksman's descriptions gives evidence —as even Waksman has partially pointed out—to the difference of proteolytic activity and, according to our opinion, the very important difference of the color of the sporulating colonies which are greenish in Krainsky's description. If we accept Waksman's correction of the species and compare his descriptions with our strains, we find a perfect identity. Since it is impossible to compare the original strain of Krainsky with the others, the acceptance of the correction proposed by Waksman offers the advantage of maintaining the name 'griseus' for an actinomycete so largely spread out and studied in laboratories, so that we agree with it according to this meaning."

The name *Actinomyces griseus* was applied by Waksman and Curtis to an organism supposedly the same as the *Actinomyces griseus* Krainsky. Later it was concluded that the two organisms belonged to distinct species. This made the binary combination *Actinomyces griseus* Waksman and Curtis a later homonym and hence not legitimate. No name was proposed to replace it.

The conclusion may, therefore, be reached that the proper designation of the streptomycin-producing organism is *Streptomyces griseus* Waksman and Henrici. The use of *griseus* as the specific epithet in the new species *Streptomyces griseus* is legitimate and correct. In support of this conclusion, the note appended to Rule 26 in the Bacteriological Code may be quoted: "Where a new epithet is required, an author may, if he wishes, adopt an epithet previously given to the taxon in an illegitimate combination, if there is no obstacle to its employment in the new position or sense; the resultant combination is treated as a new name."

Major characteristics of the S. griseus Group.—The following are the major characteristics of the *S. griseus* group:

1. Morphology: Sporophores straight, forming tufts. Spores oval to cylindrical; surface of spores smooth. 2. Aerial mycelium: Color yellowish-gray to water-green to grass green. 3. Soluble melanin pigment on protein media: None. 4. Proteolytic activities: Strong.

Species and Varieties Belonging to the S. griseus Group.—

1. *Streptomyces griseus* Waksman and Henrici. Type culture 3463. Synonyms: *Actinomyces globisporus* Krassilnikov, 1941. *Actinomyces globisporus*

subsp. *streptomycini* (Waksman) Krassilnikov, 1949. *Actinomyces streptomycini* Krassilnikov, 1957.

(*a*) Produces streptomycin; some strains also produce cycloheximide.
(*b*) Resistant to streptomycin and to streptomycin-producing cultures.
(*c*) Produces green or yellow soluble pigments on calcium malate and succinate media.
(*d*) Sensitive to a specific phage.

2. *Streptomyces griseinus* n. sp. Type culture 3478.

(*a*) Produces grisein.
(*b*) Sensitive, by cross-streak, to *S. griseus*.
(*c*) No soluble pigment on calcium malate or succinate media.
(*d*) Resistant to the common *S. griseus* actinophage.

3. *Streptomyces purpureus* (Burkholder, 1955) emend. Synonyms: *S. vinaceus* Ciba 1951; *S. puniceus* Patelski, 1950; *S. floridae* Bartz *et al.*, 1951; *S. californicus sensu* Routien and Hofmann, 1951; *S. griseus* var. *purpureus* Burkholder *et al.*, 1955.

(*a*) Produces viomycin.
(*b*) Aerial mycelium tends to be white.
(*c*) Substrate mycelium purple.
(*d*) Resistant to viomycin and streptomycin.

4. *Streptomyces coelicolor* (Müller, 1908) emend. Kutzner and Waksman.[5] Type culture available at CBS (Holland). Synonym: *S. griseus* (Krainsky) Waksman and Curtis; not *S. violaceoruber*.

(*a*) Produces an antifungal antibiotic.
(*b*) Sensitive to streptomycin.
(*c*) Some strains produce a blue pigment on potato plug and certain agar media.
(*d*) Sensitive to a specific phage.

5. *Streptomyces chrysomallus* Lindenbein, 1952. Type culture 3657.

(*a*) Produces actinomycin C; some strains also produce cycloheximide.
(*b*) Substrate mycelium on various media orange to red to purple.
(*c*) Soluble pigment on various media faint yellow to golden yellow. Var. *Streptomyces chrysomallus* var. *fumigatus* Frommer, 1959.

6. There is a sixth type culture (No. 3533) belonging to the *S. griseus* group and that is frequently found in nature. This culture produces streptocin, an antibiotic active against trichomonads, fungi, and gram-positive bacteria. This culture has not been studied sufficiently to raise it to species status.

It is interesting to note here that, in a recent publication, Koreniako and Nikitina[4] divided the *S. griseus* group into four subgroups on the basis of antibiotic sensitivity, cross resistance, and phage sensitivity. These subgroups correspond to 1, 2, 4, and 6 of the species presented here.

Descriptions of Streptomyces griseus and Streptomyces griseinus.—

Streptomyces griseus Waksman and Henrici, 1943. Type culture 3463. (Waksman and Henrici, Bergey's *Manual*, 6th ed. (1948), 948; Waksman, S. A., H. C. Reilly, and D. Harris, *J. Bacteriol.*, **56**, 259 (1948).

Morphology: Sporophores straight, produced in tufts. Spores spherical to oval, 0.8 by 0.8–1.7 μ. Surface smooth.

Sucrose nitrate agar: Growth poor, thin, spreading, colorless, becoming olive-buff. Aerial mycelium thick, powdery, water-green. Pigment insoluble.

Nutrient agar: Growth abundant, almost transparent, cream colored. Aerial mycelium powdery, white to light gray. No soluble pigment.

Glucose agar: Growth elevated in center, radiate, cream colored to orange, erose margin.

Starch media: Growth thin, spreading, transparent. Hydrolysis strong.

Potato: Growth wrinkled, yellowish to brownish. Aerial mycelium white, powdery.

Gelatin: Greenish yellow or cream colored surface growth with brownish tinge. Rapid liquefaction.

Milk: Cream colored ring; coagulation with rapid peptonization; becoming alkaline.

Cellulose: Scant to fair growth.

Nitrate: Poor utilization. Reduction to nitrite.

Production of H_2S: Negative.

Phage sensitivity: Sensitive to a specific phage.

Antagonistic properties: Strongly antagonistic. Produces an antibiotic streptomycin, active against a large number of bacteria and actinomycetes, but not against fungi and viruses, and cycloheximide active upon fungi. Resistant to streptomycin-producing organisms and to streptomycin.

Habitat: Soils, river muds, throat of chicken.

Streptomyces griseinus (Reynolds and Waksman), n. sp. (Reynolds, D. M., and S. A. Waksman, *J. Bacteriol.*, **55**, 739–751 (1948); Okami, Y., *J. Antibiotics* (Japan) **3**, 95–97 (1950).)

Morphology: Straight sporophores produced in clusters or tufts, without spirals. Spores rod shaped, 1.0–1.8 by 0.8–1.0 μ.

Sucrose nitrate agar: Substrate growth wrinkled, reverse cream colored to brownish. Aerial mycelium white to cream colored with light greenish tinge. Lesser tendency to grass-green coloration, more of a cream color. No soluble pigment.

Potato: Growth wrinkled, yellowish white. Aerial mycelium grayish white with olive tinge. Slightly brownish pigmentation of plug.

Gelatin: Growth cream colored with brownish tinge. Aerial mycelium absent, or scant, white. No soluble brown pigment. Rapid liquefaction.

Milk: Growth cream colored. Coagulation and peptonization.

Starch media: Colorless to cream colored growth. Aerial mycelium grayish olive. Rapid hydrolysis.

Tyrosine agar: No pigment produced.

Nitrate: Good utilization. Reduction to nitrite.

Phage sensitivity: Not sensitive to the phage specific against streptomycin-producing strains.

Antagonistic properties: Produces the antibiotic, grisein.

Remarks: Okami[9] reported that grisein-producing strains of *S. griseus*, here designated as a new species *S. griseinus*, grow more steadily in synthetic media containing glucose, glycerol, and sucrose than do streptomycin-producers. According to Benedict and Lindenfelser,[2] streptomycin strains of *S. griseus* form green and yellow pigments on synthetic calcium malate and calcium succinate media, respectively, whereas the grisein strains form no pigment at all on these media or only a light pink pigment.

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*COMPENSATIONS IN ELECTRON EXCITATION EFFECTS IN p-p AND p-n SCATTERING**

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Introduction.—The existence of appreciable probabilities of excitation of atomic electrons in *p-p* and *p-n* scattering caused by Coulomb Excitation and by the acceleration effect has been pointed out by Breit.[1] If the nucleon motion is treated by means of classical mechanics, these excitation effects are of little practical interest for the interpretation of *p-p* and *p-n* scattering in terms of nuclear forces, the changes in the energy and direction of the nucleons being small. The proton disappearing from the elastically scattered beam reappears with almost the same energy and at almost the same angle as an inelastically scattered one. This compensation is replaced[1] in quantum mechanics by the combination of the incoherent scattering with a modification in the coherent scattering caused by changes in phase shifts δK_L of the coherent wave. For any channel with sharp total angular momentum there appears a cross product term between the wave caused by δK_L and the usual elastically scattered wave as in Equation (2) of Reference 1. Estimates showed[1] that for the channels examined in special cases there was no compensation of incoherent by coherent effects. The possibility of the presence of nonnegligible modification in low energy *p-p* and *p-n* scattering due to quantum mechanical corrections to the excitation effects was therefore suggested.[1] On the other hand, it was pointed out[1] that the shortness of the proton and neutron wave lengths in the high energy experiments must produce agreement with classical treatment and therefore the classical mechanics type of compensation of elastic by inelastic processes.

In the present note an account is given of calculations for monopole, dipole, and quadrupole Coulomb Excitation. These were carried out according to the same general plan as that used by Breit.[1] For each of the three multipoles the effects of the different channels were combined and finally the incoherent contributions were added to the effects of the cross product terms on the coherent scattering. Employing the asymptotic expression kr-$\eta \ln 2kr - (L\pi/2) + \arg \Gamma(L + 1 + i\eta)$ for the phase of the regular Coulomb function and 1 for its amplitude the result was found to be zero. This indicates that the compensation of elastic and inelastic scattering effects present in the classical mechanics treatment carries over to some extent into the quantum mechanical problem. This circumstance was explained by Breit[2] as the result of a formal relationship between classical and

quantum mechanics which is expected to hold in the present problem on account of the smallness of the nucleon wave length in comparison with atomic dimensions. Separate action functions S_g, S_n are introduced for treating nucleon motion in the ground and nth excited states as has previously been done for tracing correspondence principle connections in nuclear Coulomb Excitation.[3] He also showed[4] that corresponding relationships in the problem of Coulomb Excitation of atomic electrons in a hydrogenic atom lead to compensations of coherent and incoherent scattering for multipole interactions of arbitrary order in the presence of an arbitrarily large finite number of nuclear phase shifts. The work reported on here may be regarded as an explicit verification of these connections in a treatment of the problem by the method of reaction channels, employing a partial wave analysis of the incident wave according to values of the orbital angular momentum L. It proved difficult to carry the calculations to the next approximation by these methods. The awkwardness in doing so lies partly in the breakdown of the asymptotic forms at high L and partly in the slow convergence of the partial wave expansion, especially at the small angles.

On the other hand, the more general approach makes it possible[2, 4] to make use of the known form for the Coulomb wave ψ^c in terms of the confluent hypergeometric function and to estimate the quantum corrections through the employment of asymptotic expansions for ψ^c. Even though the latter procedure is more expedient, it was thought of interest to report briefly on the calculations made by the reaction channel—partial wave expansion methods. The following symbols are used in the paper:

r	=	distance between incident proton and hydrogen nucleus.
ρ	=	distance between electron and hydrogen nucleus.
M	=	proton mass.
μ	=	electron reduced mass.
$v_\nu(\boldsymbol{\rho})$	=	$R_{nl}(\rho)Y_{lm}(\hat{\rho})$ = hydrogen atom wave functions, $\hat{\rho} = \boldsymbol{\rho}/\rho$ is the unit vector in the direction of $\boldsymbol{\rho}$.
ν	=	collective symbol for atomic state n, l, m.
E'	=	center of mass kinetic energy of relative motion of proton and atom.
E_ν	=	atomic energy levels.
e_L	=	$\exp(2i\sigma_L)$.
$e_{L,0}$	=	$\exp[2i(\sigma_L - \sigma_0)]$.
σ_L	=	$\arg\Gamma(L + 1 + i\eta)$.
$\begin{pmatrix} L & 1 & J \\ -\mu & \mu & 0 \end{pmatrix}$	=	vector addition coefficient, more fully explained in connection with Equation (5).
k_n^2	=	$(M/\hbar^2)(E' - E_n)$.
η_n	=	$(Me^2)/(2\hbar^2 k_n)$.
F_L, G_L	=	regular and irregular Coulomb functions, asymptotic to $\sin\theta_L$ and $\cos\theta_L$, respectively.[6]
Φ_L	=	$kr - \eta \ln 2kr - (L\pi)/2 + \sigma_L$.
H_L	=	$G_L + iF_L$.
$a_c(\theta)$	=	$-(\eta/2\mathbf{s}^2)\exp[-i\eta \ln \mathbf{s}^2]$ = Coulomb scattering amplitude.
θ, φ	=	colatitude and azimuthal scattering angles of incident proton in c.m. (center of mass) system.

Dipole Coulomb Excitation.—The problem of a proton scattered by a hydrogen atom is considered, with the simplifications of neglecting the particle spins, proton identity, and electron transfer. The hydrogen atom is taken to be in its ground state initially. In the center of mass system the Hamiltonian for the process is[5] approximately

$$H = -\frac{\hbar^2}{M}\Delta_r - \frac{\hbar^2}{2\mu}\Delta_\rho - \frac{e^2}{\rho} + \frac{e^2}{r} - \frac{e^2}{|\mathbf{r}-\boldsymbol{\rho}|}, \tag{1}$$

where $\mathbf{r}$ is the proton-proton relative coordinate vector, M the proton mass, $\boldsymbol{\rho}$ the vector displacement of the electron relative to the proton of the hydrogen atom and μ the reduced mass for relative motion in the hydrogen atom. In the present note only the Coulomb Excitation of the electron will be considered, and the cross product term of the momenta associated with $\mathbf{r}$ and $\boldsymbol{\rho}$ is neglected, since it is concerned with the acceleration effect.[1] The wave function is written as

$$\Psi(\mathbf{r}, \boldsymbol{\rho}) = \textstyle\sum_\nu \psi_\nu(\mathbf{r}) v_\nu(\boldsymbol{\rho}), \tag{2}$$

where $v_\nu(\rho)$ is the electron wave function in the atom, corresponding to the state described by the quantum numbers n, l, m,

$$v_\nu(\boldsymbol{\rho}) = R_{nl}(\rho) Y_{lm}(\hat{\rho}). \tag{3}$$

Here $\hat{\rho} = \boldsymbol{\rho}/\rho$ is the unit vector in the direction of $\boldsymbol{\rho}$.

For Coulomb Excitation of the electron from its ground state, $\nu = 1$, to the excited states $\nu > 1$, one obtains the coupled equations:

$$\left.\begin{aligned}\left[-\frac{\hbar^2}{M}\Delta_r - E' + F_1 + \frac{e^2}{r}\right]\psi_1(\mathbf{r}) &= \textstyle\sum_\nu \left(v_1, \dfrac{e^2}{|\mathbf{r}-\boldsymbol{\rho}|} v_\nu\right)\psi_\nu(\mathbf{r}) \\ \left[-\frac{\hbar^2}{M}\Delta_r - E' + E_\nu + \frac{e^2}{r}\right]\psi_\nu(\mathbf{r}) &= \left(v_\nu, \dfrac{e^2}{|\mathbf{r}-\boldsymbol{\rho}|} v_1\right)\psi_1(\mathbf{r})\end{aligned}\right\} \tag{4}$$

where E' is the kinetic energy of relative motion of the proton and hydrogen atom in the c.m. system, and E_ν is the electron energy in the hydrogen atom for the state ν.

The Coulomb Excitation term in the Hamiltonian can be expanded in multipoles, and the calculation of the dipole excitation of the electron to a p-state with principal quantum number n follows. The total wave function in this case is

$$\begin{aligned}\Psi(\mathbf{r}, \boldsymbol{\rho}) &= v_{100}(\boldsymbol{\rho})\psi_{100}(\mathbf{r}) + \textstyle\sum_\mu v_{n1\mu}(\boldsymbol{\rho})\psi_{n1\mu}(\mathbf{r}), \\ \psi_{100}(\mathbf{r}) &= \textstyle\sum_{J=L} i^J [4\pi(2J+1)e_J]^{1/2} Y_{L,0}(\hat{r}) F_{LJ}(k_1 r)/(k_1 r), \\ \psi_{n1\mu}(\mathbf{r}) &= \textstyle\sum_{J,L\neq J}\begin{pmatrix} L & 1 & J \\ -\mu & \mu & 0\end{pmatrix} i^J [4\pi(2J+1)e_J]^{1/2} Y_{L,-\mu}(\hat{r}) H_{LJ}(k_n r)/(k_n r),\end{aligned} \tag{5}$$

where $\begin{pmatrix} L & 1 & J \\ -\mu & \mu & 0\end{pmatrix}$ is a vector addition coefficient coupling angular momenta L and 1 to total angular momentum J. Their values are

$$[(2L+1)(2L+2)]^{1/2}\begin{pmatrix} L & 1 & L+1 \\ -\mu & \mu & 0\end{pmatrix} =$$

$$\{[L(L+1)]^{1/2}, 2^{1/2}(L+1), [L(L+1)]^{1/2}\}$$

$$[2L(2L+1)]^{1/2}\begin{pmatrix} L & 1 & L-1 \\ -\mu & \mu & 0 \end{pmatrix} = \{[L(L+1)]^{1/2},\ -2^{1/2}L,\ [L(L+1)]^{1/2}\},$$

the 3 numbers on the right corresponding to $\mu = 1, 0, -1$. Also $e_L = \exp(2i\sigma_L)$ and $\sigma_L = \arg\Gamma(L+1+i\eta)$. The boundary conditions are that ψ_{100} is asymptotic to a Coulomb modified plane wave + outgoing wave and $\psi_{n\,1\,\mu}$ is asymptotic to a Coulomb outgoing wave. Substitution of Equation (5) in Equations (4) results in the following set of coupled equations for the proton wave function in a state of total angular momentum J,

$$\left[\frac{d^2}{dr^2} - \frac{(J-1)J}{r^2} + k_n^2 - \frac{Me^2}{\hbar^2 r}\right] H_{J-1,\,J}(k_n r) =$$

$$-2\eta_1 \frac{k_n r}{\beta^2(r)}\left[\frac{J}{2J+1}\right]^{1/2} F_{JJ}(k_1 r),$$

$$\left[\frac{d^2}{dr^2} - \frac{(J+1)(J+2)}{r^2} + k_n^2 - \frac{Me^2}{\hbar^2 r}\right] H_{J+1,\,J}(k_n r) =$$

$$2\eta_1 \frac{k_n r}{\beta^2(r)}\left[\frac{J+1}{2J+1}\right]^{1/2} F_{JJ}(k_1 r),$$

$$\left[\frac{d^2}{dr^2} - \frac{J(J+1)}{r^2} + k_1^2 - \frac{Me^2}{\hbar^2 r}\right] F_{JJ}(k_1 r) =$$

$$2\eta_n \frac{k_1 r}{\beta^2(r)}\left\{\left[\frac{J+1}{2J+1}\right]^{1/2} H_{J+1,\,J}(k_n r) - \left[\frac{J}{2J+1}\right]^{1/2} H_{J-1,\,J}(k_n r)\right\}, \quad (6)$$

where $k_n^2 = (M/\hbar^2)(E' - E_n)$, $\eta_n = (Me^2)/(2\hbar^2 k_n)$ and $\sqrt{4\pi/3}\,Y_{1\,\mu}(\hat{r})\,r[\beta(r)]^{-2} = (v_{100}(\boldsymbol{\varrho}),\ [|\mathbf{r} - \boldsymbol{\varrho}|]^{-1}\, v_{n\,1\,\mu}(\boldsymbol{\varrho}))$. The boundary conditions require that $F_{JJ} = F_J + H_{JJ}$. The symbol F_J is used following the standard notation for the regular Coulomb function,[6] so that $F_L(kr) \sim \sin(kr - \eta \ln 2kr - (L\pi)/2 + \sigma_L)$. The coupled equations (6) were solved making use of the appropriate Green's functions and in the first two equations the factor F_{JJ} in the inhomogeneous term was approximated by F_J. The asymptotic forms of the solutions were expressed in terms of the proton orbital angular momentum L. The results are:

$$H_{L,\,L+1}(k_n r) \sim 2\,\eta_1[(L+1)/(2L+3)]^{1/2}\, H_L(k_n r) Q(L, L+1),$$
$$H_{L,\,L-1}(k_n r) \sim -\,2\eta_1[L/(2L-1)]^{1/2}\, H_L(k_n r) Q(L, L-1),$$

$$H_{L,\,L}(k_1 r) \sim 4\eta_1\eta_n(2L+1)^{-1}\, H_L(k_1 r) \times$$
$$\{L\,Q'(L, L-1) + (L+1)Q'(L, L+1)\}, \quad (7)$$

where $Q(L, L \pm 1) = \int_0^\infty F_L(k_n r)\, r\beta^{-2}(r) F_{L\pm1}(k_1 r)\,dr$,

$Q'(L, L \pm 1) =$

$$2\int_0^\infty dr \int_0^r dr' F_L(k_1 r)\beta^{-2}(r)\, H_{L\pm1}(k_n r) F_{L\pm1}(k_n r') r' \beta^{-2}(r') F_L(k_1 r'), \quad (8)$$

and $H_L(kr) = G_L + iF_L \sim \exp i(kr - \eta \ln 2kr - (L\pi)/2 + \sigma_L)$.

The integrals (8) were evaluated employing the asymptotic forms of F and H. The contributions from the high frequency parts in the integrands were neglected

and in the low frequency parts the difference between the logarithmic terms was set equal to zero. With these approximations Equations (8) reduce to:

$$Q(L, L \pm 1) \cong \pm(1/2) Im\{T[e_{L\pm1}/e_L]^{1/2}\}, \tag{9}$$

$$Q'(L, L \pm 1) \cong (i/8)\{|T|^2 + 2i\, ImT' - T^{*2}\, e_{L\pm1}/e_L\}, \tag{9'}$$

where

$$T = R + iI = \int_0^\infty \exp[i(k_1 - k_n)r][\beta(r)]^{-2}\, dr,$$

$$T' = \int_0^\infty dr \int_0^r dr' \exp[-i(k_1 - k_n)r][\beta(r)]^{-2} \exp[i(k_1 - k_n)r'][\beta(r')]^{-2}. \tag{10}$$

The quantities $e_{L\pm1}$ and e_L are to be evaluated at η_1 and η_n, respectively, in Equation (9), and η_n and η_1, respectively, in Equation (9').

The series in Equation (5) can now be summed if the difference $\eta_n - \eta_1$ is set equal to zero. The following relations were used:

$$\sum_L (2L + 1) e_{L,0} P_L(\cos\theta) = -2i\eta/(2\mathbf{s}^2) \exp[-i\eta \ln \mathbf{s}^2] = 2ia_c(\theta),$$

$$\sum_L (2L + 1) e_{L,0} P_L(\cos\theta)\, [(L + i\eta)(L + 1 - i\eta)]^{-1} = (2i\mathbf{s}^2/\eta^2) a_c(\theta),$$

$$\frac{d}{d\theta}(2i\mathbf{s}^2/\eta^2) a_c(\theta) = (\sin\theta/\eta) a_c(\theta), \tag{11}$$

where $\mathbf{s} = \sin(\theta/2)$ and θ is the c.m. scattering angle. The identifications on the right can be made when $\cos\theta \neq \pm 1$, in the limit $\epsilon \to 0$ for $\eta = \eta + i|\epsilon|$. The scattered wave is

$$\Psi_{sc}(\mathbf{r}, \boldsymbol{\varrho}) \sim r^{-1} \exp(i\Phi_1) v_{1C0}(\boldsymbol{\varrho}) s_0' + r^{-1} \exp(i\Phi_n) \sum_\mu v_{n\,1\,\mu}(\boldsymbol{\varrho}) S_\mu, \tag{12}$$

with $\Phi_n = k_n r - \eta_n \ln 2k_n r + 2\sigma_0$. The differential scattering cross section is then

$$\sigma(\theta) = |s_0'|^2 + \sum_\mu |S_\mu|^2. \tag{13}$$

The scattering amplitudes in the present approximations are obtainable from (5), (7), (9), (10), and (11) and have the values:

$$k_1 s_0' \cong \{1 - \eta^2 R^2(1 - \cos\theta) - \eta^2 I^2(1 + \cos\theta) - 2i\eta[IR\cos\theta + ImT']\} a_c(\theta),$$

$$k_n S_C \cong \{i\eta R(1 - \cos\theta) + \eta I(1 + \cos\theta)\}\, a_c(\theta),$$

$$k_n S_1 \cong \{i\eta\, R\sin\theta - \eta I \sin\theta\}\, e^{i\varphi}\, a_c(\theta);\ |S_{-1}| = |S_1|. \tag{14}$$

If the difference between k_1 and k_n is neglected in (14) the cross section is explicitly:

$$k^2\sigma(\theta) = |a_c(\theta)|^2 \{1 + \mathcal{O}(\eta T)^4\}. \tag{15}$$

The effect of a nuclear interaction in the S state has also been considered. The Equations (6) and the Green's functions for $J = 0$ and 1 are then modified. With the same approximations as above the cross section is

$$k^2\sigma(\theta) = |a_c(\theta) + \sin K_0 \exp(iK_0)|^2 \{1 + \mathcal{O}(\eta T)^4\}, \tag{15'}$$

with K_0 denoting the nuclear S wave phase shift.

The calculation of monopole and quadrupole Coulomb Excitation terms has been carried out by a similar procedure. The number of coupled equations corresponding to Equations (6) is, respectively, two and four in these cases. For the monopole

there appear terms representing screening on a static picture of the atom. These were omitted since their effects can be estimated by other methods. Otherwise the calculation is similar to that for the dipole effect and Equations (15) and (15′) obtain.

The authors would like to thank Professor G. Breit for suggesting the reaction channel method and the separation of the products of Coulomb functions into high and low frequency parts, and for stimulating discussion.

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*A SURVEY OF OUR PRESENT SOURCES OF INFORMATION ON THE CONVERSION CONSTANT, $\Lambda(=\lambda_g/\lambda_s)$ AND THE ABSOLUTE WAVELENGTHS OF X-RAY EMISSION LINES**

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Origin of the "X-Unit" and the Scale of X-Ray Wavelengths.—The wavelengths of X-ray emission lines measured relative to each other by the high precision methods of crystal diffraction are mostly known with a precision of 1 part in 10^4 to 10^5. By refined and very careful measurements with the 2-crystal spectrometer, an imprecision approaching and in some cases perhaps even smaller than a part in a million can be obtained. This precision exploration and tabulation of the X-ray spectrum was first brought to high perfection by Manne Siegbahn[1] and his school. Later, with such further improvements as the two-crystal spectrometer and the curved crystal spectrometer, it was carried even further by many others.[2–5] About 3000 of these precision wavelength measurements have been tabulated,[4] and they constitute a very reproducible and well-defined set of fixed points in the natural scale of lengths in the range between about 10^{-9} and 4×10^{-6} cm. The method employed for measuring them does not, however, yield their values directly in our cgs system of units since the interplanar grating spacings of the atomic planes in crystals are the yardsticks used. To express these wavelengths in centimeters or Angstrom units the method first adopted was that of Sir William Bragg,[6] whose reasoning was as follows.

In the simple case of the cubic structure of a rock-salt crystal, see Figure 1, each atom (sodium or chlorine) is associated with a volume, d^3, whose weight is ρd^3, where $\rho = 2.17$ gm cm^{-3} is the macroscopically measured crystal density. Now 1

mole, that is to say 23.05 + 35.45 = 58.50 gm of rock salt,† will contain $2N$ molecules, where N is the Avogadro number, so that the mean weight per atom will be $58.50/(2N)$ gm. Equating this to ρd^3, the mean weight of crystal associated with each atom, we have

$$\rho d^3 = 58.50/(2N) \tag{1}$$

$$d = (58.50)^{1/3}/(2N\rho)^{1/3} = 2.814 \times 10^{-8} \text{ cm} \tag{2}$$

provided we use for N the value Millikan obtained as the quotient of the Faraday by his oil-drop value of $e = 4.774 \times 10^{-10}$ esu. Unfortunately this latter value was about 0.6 per cent too low because in computing e from his oil-drop observations Millikan had used an erroneous value of η, the viscosity of air, as determined by Harrington. About a decade elapsed, however, before these errors were revealed.

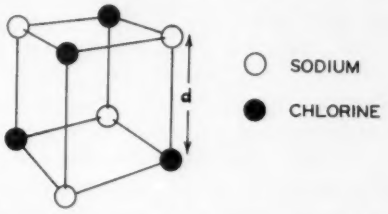

FIG. 1.—Cubic structure of a rock-salt crystal, illustrating Sir William Bragg's original method of computing the grating space, d, given the Avogadro number, the crystal density, and its molecular weight.

The structure of rock salt is less perfect and less reproducible from sample to sample than certain other crystals; calcite, for example. In 1925 Compton, Beets, and DeFoe[7] made very careful measurements on the latter rhombohedral crystal to determine its density and the angles between its cleavage planes. The latter goniometric measurement permits calculation of the volume of a calcite rhombohedron having unit separation between its three pairs of parallel faces. They found for this volume, $V = 1.09630 \pm 0.00007$. (In later work J. A. Bearden[28] obtained a slightly different numerical value.) As a final result of their calculation, whose principle is the same as that just described for rock salt, they obtained for the true grating space a value which M. Siegbahn *adopted* as the basis for calculating X-ray wavelengths. This calculated grating space was, however, erroneous chiefly because of the error in Harrington's value of η with its consequent effects on e, and thence on the value of $N = F/e$ which Compton, Beets, and DeFoe had used.

In view of these errors it is best to regard Siegbahn's system of wavelengths as a purely arbitrary, albeit highly reproducible system, and indeed Siegbahn *very wisely* chose to call the unit length in that system by a distinctive name, the x-unit, for although he had certainly intended to make the x-unit as nearly as possible equal to a milliangstrom, it seems highly likely that from the beginning he felt more insecurity about its absolute value than was comfortable in view of the high precision with which X-ray wavelengths could be measured relative to each other by means of crystals. For many years no distinction between x-units and milliangstrom units was suspected. The terms were used interchangeably. Now that the discrepancy between them is well established it is customary merely *to define the x-unit* as Siegbahn and the rest of us have come to do, by the statement that the "effective"‡ grating space of the calcite cleavage planes at 18°C for first order Bragg reflection is

$$d_{18}'' = 3029.040 \text{ x-units} \quad (3)$$

The author believes, for reasons to be discussed in this paper, that it would be still better to define the x-unit in terms of at least one or perhaps more carefully chosen standard X-ray emission lines. This point will be discussed in due course.

$\Lambda(= \lambda_g/\lambda_s)$ *the Ratio of the X-Unit to the Milliangstrom Unit.*—The discrepancy between Siegbahn's x-unit and the milliangstrom unit first began to be manifest when the wavelengths of certain soft X-ray lines which had been measured in x-units by the methods of crystal diffraction were also precisely measured with artificially ruled gratings whose grating constants had been calibrated using known or calculable optical wavelengths. Apparently the first to note the discrepancy was Erik Bäcklin in his thesis dissertation at Uppsala (1928).

Because of the deservedly great prestige of R. A. Millikan and the rather obscure nature of the true source of error, more than a decade elapsed before there was general acceptance that Millikan's value of e was seriously erroneous. During this period there was much discussion of the "discrepancy" between the "oil-drop" and the "X-ray" values of e. Several rather far-fetched proposals to explain this discrepancy were made by proponents of the Millikan value before the error in η, the viscosity of air, was definitely established as the chief source of the trouble: (1) It was proposed that a mosaic structure or that impurities in the crystals might be responsible. (2) It was suggested that the laws of optics might not be applicable to the diffraction of X-rays from ruled gratings at grazing incidence; a careful analysis by C. Eckart refuted this. (3) The grating constant measured with X-rays might not be the same as the grating constant throughout the bulk of the crystal lattice. This third criticism was the most tenacious and the most difficult to refute. The Bragg reflection from atomic planes parallel and very close to a boundary surface had always been used in precision determinations, and since the surface is admittedly a highly specialized locality, the atomic structure and hence the grating constant might conceivably have a special value there. (An analogy might be made with surface tension in liquids.) The macroscopic density measurements of a crystal used by Sir William Bragg, A. H. Compton, or M. Siegbahn in their method of computing the grating constant, on the other hand, clearly must yield the average grating constant over the entire sample of crystal. The plausibility of this criticism and also that of the first one was greatly weakened by showing[8] that the measured densities and X-ray values of grating constants combined with molecular weights gave, for many different kinds of crystals of the perfect type, mutually consistent values of the Avogadro number, N. (Reversing the Bragg method of calculating crystal grating constants, if we know from X-ray measurements the grating constant, d, of a crystal, its density, ρ, the molecular weight, M_μ, of its unit cell, and the dimensionless geometrical shape factor, ϕ, the volume of a unit cell whose grating constant, d, is unity, we can calculate Avogadro's number, N, from $N = M_\mu/(\rho\phi d^3)$. The third criticism persisted for a time nonetheless because of a suggestion by F. Zwicky that a superlattice structure, consisting of periodic local deviations of the lattice from the average grating spacing, might be a general property of all crystal lattices because of some very fundamental far-reaching and general "cooperative" effect extending over wide domains. Zwicky suggested that the boundary surface of a crystal might, by some considerations of

minimum energy, be invariably the site of one of these abnormal grating constant regions. The third criticism was removed entirely, however, by two methods: (*a*) X-rays were reflected from crystals of the perfect type such as calcite using *internal* atomic planes for this purpose and the internal grating constant was shown to be the same as at the surface;[9, 10] (*b*) Crystals such as calcite and quartz were powdered so finely that the penetration of X-rays through each grain was complete and not appreciably limited by "extinction." The grating constant for the powder was then measured with high precision using a Seeman-Bohlin spectrograph, and the density of the identical sample of powdered crystal was measured with a pycnometer. No significant deviation was observed in the inferred value of N from that obtained with macroscopic crystals used in Bragg surface reflection.[9, 11] Several experimeters have repeated the oil-drop experiment and also the determination of the viscosity of air and it is now quite clearly established that no real discrepancy exists between the X-ray and oil-drop values of e.

Save for the case of extremely long X-ray wavelengths the great bulk of the ruled grating measurements of X-ray lines have been made using plane gratings by the method of grazing incidence. Compton and Doan[12] and shortly afterward Thibaud[13] were the first to use this method. In grazing incidence far greater dispersion is obtainable with a grating of a specified number of rulings per unit distance than in the more familiar case of normal incidence.

A condition for sufficient intensity in the diffracted spectrum is that the angle of grazing incidence shall be less than the critical angle for total reflection. It should be recalled that the refractive indices of X-rays are in most cases less than unity. Total reflection occurs therefore in the less dense medium. Rather coarse gratings with a fairly large ratio of flat intervening space to the width of the ruled lines are usually used and methods of ruling which are likely to produce a projection of the ruled material above the flat intervening surfaces are to be avoided.

The geometry controlling the path difference, $PB\text{-}AR = a[\cos\theta - \cos(\theta + \alpha)]$, is shown in Figure 2. It must be recalled that for X-rays no lenses are available to

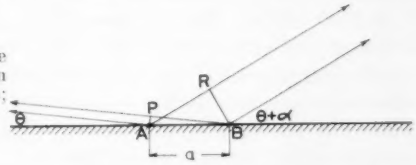

FIG. 2.—Illustrating the geometry of the path difference when ruled gratings are used in grazing incidence to measure X-ray wavelengths; a is the grating spacing.

render a divergent beam parallel or to focus a parallel beam to a point, as in the case of ordinary optical light. Setting the path difference equal to a whole number of wavelengths, $n\lambda$, we have

$$n\lambda = a[\cos\theta - \cos(\theta + \alpha)]. \tag{4}$$

In the simplest of all cases when θ can be regarded as negligible we have

$$n\lambda = a(1 - \cos\alpha) \cong a\alpha^{2}/_{2}. \tag{5}$$

The dispersion for grazing incidence, $(d\alpha/d\lambda)_g$, is

$$(d\alpha/d\lambda)_g = \sqrt{n/(2a\lambda)}. \tag{6}$$

This is to be compared with the dispersion for normal incidence, $(d\alpha/d\lambda)_n$, which is

$$(d\alpha/d\lambda)_n \cong n/a, \text{ for small } \alpha. \tag{7}$$

The chief precision wavelength measurements of X-ray characteristic spectral lines by diffraction on ruled gratings to date have been made by four authors, J. A. Bearden,[14] E. Bäcklin,[15] and M. Söderman,[16] and F. Tyrén,[17] between 1931 and 1938. The softer X-ray lines CuKα, β, AlKα were chosen for study. The results are best expressed in terms of the conversion factor $\Lambda = \lambda_g/\lambda_x$ implied by the measurements and these will be found in Table 1.

TABLE 1

COMPARISON OF GRATING AND SIEGBAHN WAVELENGTHS OF X-RAYS (1945)

Author	X-Ray Line	$(\Lambda - 1) \times 10^6$ (The Probable Error Is Based on Accidental Errors Only)	Adopted Probable Error
Bearden*	CuK$\alpha_{1,2}$	1980 ± 31	±52
Bearden*	CuK$\beta_{1,3}$	2079 ± 28	±50
Bearden*	CrK$\alpha_{1,2}$	2036 ± 21	±48
Bearden*	CrK$\beta_{1,3}$	2017 ± 28	±48
Söderman†	AlK$\alpha_{1,2}$	2070 ± 37	±84
Bäcklin‡	AlK$\alpha_{1,2}$	2000 ± 14	±60
Bearden§	CuK$\alpha_{1,2}$	2087 ± 55	±130
Tyrén‖	AlK$\alpha_{1,2}$	2024 ± 5	±48
	Adopted average	2030 ± 20	

* Bearden, J. A., *Phys. Rev.*, **37**, 1210 (1931).
† Söderman, M., *Nature*, **135**, 67 (1935); Dissertation, Uppsala, 1934.
‡ Bäcklin, E., *Z. Physik*, **93**, 450 (1935).
§ Bearden, J. A., *Phys. Rev.*, **48**, 385 (1935).
‖ Tyrén, F., *Z. Physik*, **109**, 722 (1938).

The measurements of Λ in Sweden were chiefly done using concave grating vacuum spectrographs with grazing incidence angles on the grating of order 1°. Perhaps the best and most carefully performed of these were the measurements of F. Tyrén in his dissertation.[17] His method consisted in recording on one and the same Schumann plate, bent to conform to the Rowland circle of his instrument, two sets of lines: (1) Calibration lines consisting of Lyman Series spark lines from one-electron (hydrogenic) ionized atoms, O^{VIII}, N^{VII}, C^{VI}, B^{V}, and Be^{IV} and (2) soft X-ray lines which had already been measured using crystals and were therefore known on the "crystal scale" in x-units and were to be determined by interpolation from the calibration lines on the nonlinear wavelength scale of the plates. Tyrén computed the wavelengths of the calibrating spark lines in angstrom units using the very accurately known value of the Rydberg and theoretical spectroscopic formulas based on the Bohr-Sommerfeld-Dirac theory for the hydrogen-like atom. At the time Tyrén wrote his thesis the Bohr-Sommerfeld-Dirac theory was considered absolutely correct, but a few years later the advent of the Lamb shift and its explanation in terms of modern quantum-electrodynamics called for a correction to the Bohr-Sommerfeld-Dirac theory, the magnitude of which is different and far from negligible for each of Tyrén's calibration lines. Although the need for this correction to Tyrén's result was (by their own later admission) recognized by some of the Swedish school of spectroscopists as soon as Lamb's discovery was published, no attention was publicly called to it and it went completely unnoticed until as late as 1956 when the present writer and E. R. Cohen published a note[18] about it. Table 2 shows these Lamb shift corrections calling for increases in the calibration

TABLE 2

RELATIVE INCREASES REQUIRED TO CORRECT TYRÉN'S REFERENCE WAVELENGTHS FOR THE LAMB SHIFT

Tyrén's calibration wavelengths, Lyman series lines of O^{VIII}, N^{VII}, C^{VI}, B^{V}, Be^{IV} were calculated using a formula in conformity with the Sommerfeld-Dirac theory of the one-electron atom. The Lamb shift corrections, $\Delta E/E$, to this formula in ppm are:

n		O^{VIII}, ppm	N^{VII}, ppm	C^{VI}, ppm	B^{V}, ppm	Be^{IV}, ppm
1	Lyman alpha	98.5	81.5	64.5	49.0	34.5
2	Lyman beta	84.0	69.0	54.7	41.0	29.0
3	Lyman gamma	79.0	65.5	52.0	39.1	27.5
4	Lyman delta	77.5	63.5	50.4	38.3	26.8

Note: For $np - 1s$ transitions $\Delta E/E = \{8n^2/[3\pi(n^2 - 1)]\}(7.723 - 2 \ln Z - 0.0439/n^3)$.

wavelengths which vary from 98.5 parts per million (ppm) to 26.8 ppm. Unfortunately Tyrén's thesis does not specify just how the calibration lines were associated with the X-ray lines and since both sets of lines were recorded in many different orders (up to the 13th in the case of some X-ray lines) one cannot conclude that X-ray lines and spark lines most closely adjacent in wavelength were necessarily those associated for the purpose of calibration. It is to be hoped that Tyrén's original notes and calculations and also his original plates can be secured and corrected for the Lamb shift but no such correction has as yet been made. Tyren's result, $\Lambda = 1.00199$ (which he stated without giving any estimated standard deviation), must certainly therefore be increased by some amount between 26.8 and 98.5 ppm. A rough estimate by E. R. Cohen based on the assumption that those calibration spark lines and X-ray lines most nearly adjacent in wavelength were associated, yields a corrected average value for Tyrén's determination of $\Lambda = 1.002026 \pm 0.000016$. Little significance can be attached to this, however, save to show that the correction certainly seems to promise better consistency between Tyrén's results and those obtained chiefly by J. A. Bearden in the United States using plane ruled gratings.

Importance of the Conversion Constant, Λ, in the Determination of the Entire System of Constants and Conversion Factors of Physics and Chemistry.—The importance of the conversion constant, Λ, in contributing to our knowledge of *all of the fundamental constants of atomic physics*, though little realized, can hardly be overstated since it is the bridge between our tangible macroscopic scale of length magnitudes and the scale of atomic length magnitudes. Because of the many intricate relations which now link all of the fundamental atomic constants of Physics and Chemistry a revision in the directly measured value of Λ would have extremely for reaching effects on our present state of knowledge of all of the fundamental constants. For example in our 1955 least squares evaluation of the constants,[19] far each part per million change in the directly measured input value of Λ, the resulting change in parts per million which eight important output constants would sustain is shown in Table 3.

It must not be supposed that the differential coefficients whose numerical values are given in Table 3 are constants of nature. They are bound to change with every new least-squares adjustment to obtain "best" values because they depend on which functions of the atomic constants were those whose measured values constituted the input data of the particular adjustment and also on the precision with which those measurements were made. Both of these things are altered from their

previous state at every epoch when a new adjustment is effected, because of the progress of measuring techniques, and indeed a new adjustment would not be warranted unless such changes had occurred. However, it is hopeless to anticipate a time when such interrelationships will vanish.

TABLE 3

CHANGES, IN PPM, SUSTAINED BY EIGHT IMPORTANT OUTPUT VALUES OF ATOMIC CONSTANTS AS A RESULT OF A CHANGE OF 1 PPM IN THE DIRECTLY MEASURED INPUT VALUE OF Λ IN THE COHEN, DUMOND, LAYTON, AND ROLLETT LEAST SQUARES ADJUSTMENT OF 1955

e	0.14
m	0.22
h	0.26
α	0.02
Λ	0.21
N	−0.21
F	−0.07
$hc^2/(e\Lambda)$*	−0.08

* This is the voltage-wavelength conversion factor whose adjusted value as an output result of the 1955 adjustment, was 12372.44 ± 0.16 kv X-units. Dividing this number by any wavelength expressed in X-units gives the corresponding quantum energy in electron-kilovolts.

Proposal to Express the International Crystallographic Wavelength Tables in Milliangstrom Units; Advantages and Disadvantages.—The present survey of the state of our knowledge of Λ is of considerable current interest and was undertaken chiefly because of a proposal now very seriously being considered on the part of crystallographers, particularly for the preparation of the new International Tables of Crystallography, to tabulate all the wavelengths of the standard X-ray emission lines in angstrom units rather than as heretofore in x-units on the crystal scale. Those who favor this step at the present time do so because they believe it will lead to simplification and will avoid a great deal of confusion now assertedly resulting from ignorance on the part of some crystallographers of the distinction between the x-unit and the milliangstrom unit. The author of this paper would only agree with this point of view if it were possible to establish beyond all reasonable doubt the conversion from the x-units of the crystal or Siegbahn scale, in terms of which the X-ray lines were measured, to milliangstrom units with the same or preferably with better precision than the precision with which the X-ray line measurements were made on the x-unit scale. For, if this is not possible, then either (1) the tabulation of all the X-ray reference line wavelengths in terms of numbers labeled angstrom units must be seriously down-graded to a precision far poorer than the best available precision with which X-ray wavelengths are known relative to each other to allow for the imprecision in our knowledge of the single conversion constant afflicting all of the data, or (2) some conventional value of the conversion constant must be adopted by general consent to which a fictitiously high precision is assigned. But in this last case we shall not in reality be telling the truth if we label the numerical values so computed as values expressed in milliangstrom units for we shall be doing once more (on a different level of precision, it is true) exactly the same thing which was done when the x-unit was established with the aim of making it equal to a milliangstrom. Hence, sooner or later, we shall surely discover that instead of simplifying the situation and avoiding confusion, we have increased the confusion by printing tables which are expressed in terms of a unit which is neither exactly a milliangstrom nor an x-unit and which for definiteness will thenceforth

have to be given a name. Such a unit might well deserve to be named "The Folly of the Crystallographic Congress of 1959."

The temptation to circumvent a real difficulty of physical measurement by introducing a conventionally defined conversion constant, so prevalent at International Congresses, is becoming increasingly important to resist because of the ever increasing tightness with which all the different quantities of Physics and Chemistry are interrelated. The specialist in a given field is not likely to be as aware of the intricate tightness of these interrelationships as are those, like Dr. Cohen and myself, who have concerned themselves for a long period of time with efforts to distill from the entire mass of information in both Physics and Chemistry the best values of all the constants of those related disciplines. Such a task can no longer be tackled piecemeal. The two disciplines cannot be treated separately. The entire system is like an immense cobweb. You pull on any of the filaments and the whole pattern changes to some extent. Starting from the present wealth of observational data there are no longer any well marked single routes by which to arrive at values of a specified constant or conversion factor. Each desired result can be arrived at by a multiplicity of different single-track (just-determinate) routes no one of which is by any criterion so greatly to be preferred to all others as to indicate it indubitably for acceptance. We have no escape in this situation save through very comprehensive least-squares analyses very carefully combed in an effort to detect and cast out systematically erroneous data. Unless the practitioners of each specialized field, crystallography, thermodynamics, X-rays, nuclear physics or what you wish, realize that they can no longer regard their particular discipline as an area isolated from the rest of Physics and Chemistry, the beautiful edifice which together the builders of these two divisions of science have constructed will become a Tower of Babel.

How Accurately Can X-Ray Wavelengths be Expressed in Millingstrom Units by Direct Determinations of Λ?—It is for the foregoing reason that the author of this paper deems it timely and desirable to examine with some care just how much we presently know, from all sources of information, both direct and indirect, about this conversion constant, $\Lambda\ (= \lambda_g/\lambda_s)$.

Table 1 above, prepared by R. T. Birge[20] in 1945, shows the directly measured data on Λ from all sources then available, both the flat grating work of Bearden, in which no calculated calibration wavelengths were needed because the incidence and reflection angles were directly measured, and the Swedish concave grating work. Tyrén's 1940 dissertation results, which were afflicted with the systematic error from the Lamb shift as above explained, are not included in this tabulation but it seems likely that the 1938 work of Tyrén which is shown in the last item suffers from the same error; but to what extent we do not know. This single item, if corrected, would probably have little effect on Birge's final weighted average which was:

$$\Lambda = 1.002030 \pm 0.000020 \text{ (Birge, 1945)} \qquad (8)$$

This value agreed (save for a larger assigned error measure) with a weighted average obtained earlier by J. A. Bearden.[21] The Birge 1945 value was accepted and used until in 1947 Sir Lawrence Bragg issued an ex-cathedra statement which appeared in several journals widely read by crystallographers in England and the

United States[22] to the effect that "after consultation with Siegbahn, Warren, and Lipson, and with due consideration given to the above data listed by Birge," he recommended for general adoption the value

$$\Lambda = 1.002020 \pm 0.000030 \text{ (Bragg edict of 1947).} \quad (9)$$

No statement of any basis in new physical measurements was given to justify this edict. The author of this paper could obtain no clear explanation from Sir Lawrence Bragg or for a long period from any one else as to just how this value had been arrived at and in particular why Birge's 1945 average had been deliberately altered, until in 1956 he happened to broach the subject with R. T. Birge himself, who it seems was much more involved in the controversy and compromise than the innocuous wording of the edict first led the present writer to suppose. It appears that the chief argument for a downward revision in 1947 of Birge's average value was due to M. Siegbahn who urged it because of the result of Tyrén's dissertation, Uppsala 1940,

$$\Lambda = 1.00199 \text{ (Tyrén, Dissertation 1940)} \quad (10)$$

to which no error estimate had been attached by its author. M. Siegbahn at the time was, however, so impressed with the excellence of this work (and indeed it was beautifully executed, though the details of the interpolation were not as clearly revealed in the dissertation as one could wish) that he agitated strongly for a revision downward of Birge's value. Siegbahn had at the time, of course, no way of foreseeing the discovery of the Lamb shift three years later. Birge, on the other hand, chiefly because of the convincing nature of Bearden's results with plane gratings, refused to consent to lowering of his weighted average of 1945 by more than one unit in the fifth decimal place and would accept that only if a correspondingly greater spread were assigned in the error measure. Actually, if, as, and when Tyrén's observations and calculations can be corrected for the Lamb shift, it now appears not unlikely that the apparent discrepancy between Tyrén's 1940 result above and Birge's weighted average of 1945 will be essentially wiped out. The world therefore probably owes Birge a debt of gratitude for his refusal to modify Λ more drastically. After a lengthy period of correspondence, in the full knowledge of a serious unexplained discrepancy and without any published explanation of the doubtful procedure, the value given in the Bragg edict of 1947 was recommended and promulgated. Such authoritarian practices (publishing a recommendation for adoption of an important conversion constant *with no detailed public statement of reasons or experimental bases*) is greatly to be deplored as completely contrary to the spirit of science![§]

It appears that though the Swedish school of precision X-ray spectroscopists made no effort to warn the rest of the world of a systematic error in the results of Tyrén on Λ after the Lamb shift had become an established fact, they did feel sufficiently insecure about Tyrén's $\Lambda = 1.00199$ to continue energetically a program directed at bettering this determination and several pieces of work about which little has been published have been performed. For example, an unpublished recent piece of work of Björkman at Uppsala following the same method as Tyrén has yielded a surprisingly high value

$$\Lambda = 1.00216 \pm 0.00004 \text{ (Björkman unpublished)} \quad (11)$$

140 ppm higher than the Bragg edict of 1947! Björkman's manuscript does not make it clear whether the Lamb shift was taken into account in his calculations.

If we examine Birge's Table 1 we see that the error estimate for the weighted average calculated by the methods of statistics (valid when the errors are purely random) arrives at a "probable error" of only 20 ppm. Actually the eight individual values averaged wander all the way from 1.001980 to 1.002087, a spread of 107 ppm and one should therefore not be too surprised or disappointed to discover later that systematic errors may well have been present which distorted the average considerably more than the statistically computed error.

Because of the great importance of this constant, Λ, and because the uncertainties here discussed really throw this question wide open once more, redeterminations of its value are now being undertaken in two places and it would be highly desirable if similar projects could be started elsewhere, preferably in publicly supported government standards laboratories where the expense of a long program of extremely careful work on such a determination can be more logically justified and supported. One of these two presently going programs on Λ is now in progress by Professor H. A. Kirkpatrick, with the collaboration of the present author, utilizing the method of Tyrén, with a concave grating of 42 foot radius of curvature in a vacuum spectrometer, the lines being recorded on Schumann plates. Professor Kirkpatrick is doing this work at the present moment at the University of Wisconsin as a guest in the laboratory of Professor Julian Mack to whom we are deeply grateful for the use of his vacuum spectrometer. The other program, I am told, is being pursued by Professor J. A. Bearden and his son at Johns Hopkins University utilizing plane gratings, in vacuum, the X-rays incident on the grating being first monochromatized by crystal diffraction. At the date of writing no definite results are available from either of these sources.

Indirect Information on the Value of Λ.—It is well worth while now to examine exhaustively just what indirect information on the value of Λ can be inferred from presently existing data of measurement. This can be conveniently done by considering all the input data, which were used in the 1955 adjustment of the fundamental constants of Cohen, DuMond, Layton, and Rollett[23] taking into account as far as possible such changes and corrections and new sources of information as have become available subsequently. The four primary unknowns, α, e, N, and Λ, whose least-squares adjusted best values were sought, together with the two groups of experimental data used in setting up the equations for the least-squares solution, are listed and defined in Table 4. In the first group, seven kinds of functions of the unknowns appear equated to their experimentally determined numerical values. Each equation is labeled with the experiment which leads to it. It must be understood, however, that in order to utilize the results of many of these experiments in equations involving only the four chosen unknowns, it has been necessary to combine with the experimentally measured quantity certain other extremely accurately known constants whose values are listed in the second group and whose accuracy so greatly surpasses the accuracy of the measurements listed in the first group that they do not contribute appreciably to the error of the result. Thus the "measured numerics" in the first group involve, for their computation, not only the experimental measurements listed under the heading "Description of Experiment," but also appropriately selected data from the second group requisite to give the

TABLE 4

RÉSUMÉ OF THE DATA CONSIDERED FOR THE 1955 LEAST SQUARES ADJUSTMENT OF COHEN, DuMOND LAYTON, AND ROLLETT

Four primary unknowns whose least squares adjusted values are sought:

- α ($= 2\pi e^2 h^{-1} c^{-1}$), Sommerfeld's fine structure constant.
- e, electronic charge (expressed in abs. esu).
- N, Avogadro number.
- Λ ($= \lambda_g/\lambda_x$), conversion factor from X-units (Siegbahn) to milliangstroms.

First Group (Least Squares Adjustment)
Seven Kinds of Experimental Data Bearing on Above Unknowns

Kind No.	Function	Measured Numeric	Estimated Precision (ppm)	Description of Experiment
1	Ne/c	$= 9652.15$	13	Iodine } Faraday by electrochemistry
	Ne/c	$= 9651.29$	20	Silver }
2	Λ	$= 1.002020$	30	X-ray lines measured with ruled gratings
3	$N\Lambda^3$	$= 0.606179 \times 10^{24}$	38	Crystal densities and grating constants in X-units
4	$\alpha^3 c/e$	$= 2.425517 \times 10^{13}$	23	Proton Gyrom. Ratio (Thomas, Driscoll, & Hipple)
5	$Ne^2/(\alpha^3 c^2)$	$= 3.979879 \times 10^{-10}$	36	Bloch & Jeffries, "Inverse cyclotron" } Proton magn. moment in nuclear magnetons by measuring ω_c/ω_p
	$Ne^2/(\alpha^3 c^2)$	$= 3.979423 \times 10^{-10}$	11	Sommer, Thomas & Hipple, "Omegatron"
	$Ne^2/(\alpha^3 c^2)$	$= 3.979359 \times 10^{-10}$	14	Collington, Dellis, Sanders, & Turberfield
	$Ne^2/(\alpha^3 c^2)$	$= 3.979444 \times 10^{-10}$	36	Kenneth R. Trigger's corr. to B & J
6	$\alpha^2 c$	$= 1.596412 \times 10^6$	9	Microwave determination of fine structure splitting in deuterium — W. Lamb, Jr., and coworkers
7	$ec/(\Lambda\alpha)$	$= 1968.750$	51	Felt, Harris, & DuMond } Short wavelength limit of continuous X-ray spectrum
	$ec/(\Lambda\alpha)$	$= 1968.911$	40	Bearden, Johnson & Watts
	$ec/(\Lambda\alpha)$	$= 1968.869$	83	Bearden & Schwarz

Second Group (Regarded as Fixed Constants)

H	$= 1.008142 \pm 0.000003$ (Physical)	3	Atomic weights by method of nuclear reaction energies
H/M_p	$= 1.00054461$		
D	$= 2.014735 \pm 0.000006$ (Physical)	3	
D/M_d	$= 1.00027244$		
R_∞	$= 109737.309 \pm 0.012$ cm^{-1}	0.11	Spectroscopic determination of R_∞
μ_e/μ_p'	$= 658.2288 \pm 0.0004$	0.6	Ratio electron magnetic moment to proton magnetic moment
μ_e/μ_0	$= 1.00114536$	<0.01	Ratio electron magnetic moment to Bohr magneton
c	$= 299793.0 \pm 0.3$ km sec^{-1}	1	Velocity of light

numeric the significance indicated by the left-hand member of each equation.

The seven kinds of equations of Table 4, comprising a total of thirteen equations in four unknowns, are called "the primitive equations of observation." They constitute a highly overdetermined set whose mutual compatibility turned out to be considerably poorer than the *a priori* estimates of precision (shown in Table 4) would lead one to expect, thus casting strong suspicion that some members contain systematic errors.

Such a set is most conveniently adjusted and analyzed as to its compatibility after a "linearization" procedure as follows: A set of "origin values," α_0, e_0, N_0, and Λ_0, can easily be chosen for the unknowns which will lie within a few tens of parts per million of the least-squares adjusted values to be found. The origin values chosen were

$$\alpha_0 = 0.007297000 \tag{12}$$

$$e_0 = 4.802200 \times 10^{-10} \text{ esu} \tag{13}$$

$$N_0 = 0.6025000 \times 10^{24} \text{ mole}^{-1} \tag{14}$$

$$\Lambda_0 = 1.0020200 \tag{15}$$

We then re-express the unknowns and the constants of the thirteen equations of Table 4 in terms of new variables, $x_1 = 10^5 (\alpha - \alpha_0)/\alpha_0$; $x_2 = 10^5 (e - e_0)/e_0$, etc., and new constants $A = 10^5 (c - c_0)/c_0$ where c is the constant in the particular equation and c_0 the origin value that constant would have in order to be compatible with the four origin values selected for the unknowns, in other words we express the entire set in terms of small relative deviations in parts per hundred thousand from an arbitrary origin point in the space of the four unknowns, a point selected so as to lie close to the final solution. This is essentially the same as expressing the equations in terms of the logarithms of all the quantities and in this new convenient linearized form one obtains a set of linear equations of which we will only write out a few samples as illustrations:

		Weight	Description of Experimental Source
$x_2 + x_3 = 11.1$		0.58	(Iodine) Faraday by electro-chemistry
$x_4 = 0$		0.11	$\Lambda = 1.002020$, Bragg edict
$x_3 + 3x_4 = 3.5$		0.07	Crystal densities and grating constants in X-units
$3x_1 - x_2 = -2.3$		0.19	Thomas Driscoll & Hipple gyromagn. ratio of proton

These then are the overdetermined linear equations to which the well-known classical procedure of least squares is applied to determine a set of "adjusted values" for the unknowns. The measure of compatibility used is called χ^2 and is defined as the sum of the squares of the normalized residues of all the linearized equations. The residue of an equation is the amount by which it fails to balance when the least-squares adjusted values of the unknowns are substituted in it and the normalized residue is the actual residue divided by the *a priori* standard deviation attached to the numeric of that equation. χ^2 is in fact the minimum value of the quantity which it is the objective of the method of least squares to minimize. In the present instance χ^2 turned out to be 52.1 whereas its expected value (in the absence of systematic errors) should be 8. A search for suspiciously incompatible equations was then made by an elaborate digital computer program described in detail in the 1955 paper[23] and in this way six equations were spotted and, in the case of each, valid reasons for rejection were adduced because sources of systematic error could be shown to be either definitely present or highly probably for good and sufficient physical reasons. The final set of linearized equations after this censorship was accomplished had a measure of compatibility, $\chi^2 = 3.25$, the expected value in absence of all systematic error being $\chi^2 = 3$ which is not a statistically significant difference in this case. Three years after the 1955 adjustment was completed an error was discovered in the theoretically calculated value of Karplus and Kroll[24] of μ_e/μ_0, the next to the last datum in the group of fixed constants listed in Table 4. As we pointed out in a recent letter to the *Physical Review*,[25] the change of 14.3 ppm in μ_e/μ_0 modifies the numerical values in the case of three of these equations, the third, the fourth, and the eighth. After effecting this modification the seven linearized observational equations take the form shown in Table 5. These equations of Table 5, then, constitute the starting material from which we shall derive all indirect values of Λ implicit in them. The first equation results of course from *direct* measurements of Λ already discussed and we shall therefore make no use of it.

TABLE 5

THE LINEARIZED OBSERVATIONAL EQUATIONS ON WHICH THE 1955 L. S. ADJUSTMENT WAS BASED AFTER CORRECTION OF THE 14.3 PPM ERROR IN μ_e/μ_0

					Weight	Description of Experimental Source	
		x_4	$= 0 = A_1,$		0.11	$\Lambda = 1.002020$	(16)
	$x_3 +$	$3x_4$	$= 3.5 = A_2,$		0.07	$N\Lambda^3$ (Birge's average)	(17)
x_1			$= 2.6 = A_3,$		4.92	Dayhoff fine structure splitting in deuterium	(18)
$3x_1 -$	x_2		$= -3.7 = A_4,$		0.19	Thomas, Driscoll, and Hipple gyromagnetic ratio of proton	(19)
	$x_2 + x_3$		$= 11.1 = A_5,$		0.58	(Iodine) Faraday by electrochemistry	(20)
$-3x_1 + 2x_2 +$	x_3		$= 14.9 = A_6,$		0.83	Sommer, Thomas and Hipple (omegatron) mag. moment of proton	(21)
$-x_1 +$	x_2	$-x_4$	$= -5.6 = A_7,$		0.015	Short wavelength limit of continuous X-ray spectrum. Mean of low-voltage values	(22)

The remaining six equations can be combined in nine sets of four equations each and one set of three equations, each of the ten sets being just sufficient to obtain a different just-determinate solution for Λ. Table 6 displays these ten solutions and gives the ten numerical results with their standard deviations. Each solution is labeled with a set of numbers (e.g., 2345) which designate which of the constants in Table 5, A_2 to A_7 inclusive, entered into that solution. The writer believes there are no other just-determinate solutions than these ten. While all these solutions are *different*, it must not be supposed that they are *independent* of each other.

TABLE 6

COMPUTATION OF TEN DIFFERENT JUST-DETERMINATE INDIRECT SOLUTIONS FOR Λ FROM THE DATA OF TABLE 5

(The Corrected value of μ_e/μ_0 along with the γ of T. D. & H. are used)

	t		Parts in 10^6	σ, Ppm	Λ
(1)	$x_4 = {}^1/_3(A_2 + 3A_3 - A_4 - A_5)$	$=$	0.967	16.05	1.002030 ± 0.000016
(2)	$x_4 = {}^1/_3(A_2 + 3A_3 - 2A_4 - A_6)$	$=$	1.267	20.7	1.002033 ± 0.000021
(3)	$x_4 = 2A_3 - A_4 - A_7$	$=$	14.5	85.	1.002165 ± 0.000085
(4)	$x_4 = {}^1/_3(A_2 + 3A_3 - 2A_5 + A_6)$	$=$	1.33	16.5	1.002033 ± 0.000017
(5)	$x_4 = {}^1/_2(A_2 + A_3 - A_5 + A_7)$	$=$	-5.3	45.5	1.001967 ± 0.000046
(6)	$x_4 = A_2 - A_3 - A_6 + 2A_7$	$=$	-25.2	168.0	1.001768 ± 0.000168
(7)	$x_4 = {}^1/_3(2A_2 + A_4 - 2A_5 + 3A_7)$	$=$	-11.9	86.2	1.001901 ± 0.000086
(8)	$x_4 = {}^1/_3(2A_2 - A_4 - 2A_6 + 3A_7)$	$=$	-11.97	86.0	1.001900 ± 0.000086
(9)	$x_4 = {}^1/_3(2A_2 - A_5 - A_6 + 3A_7)$	$=$	-11.93	85.6	1.001901 ± 0.000086
(10)	$x_4 = 2A_3 - A_5 + A_6 - A_7$	$=$	14.6	112.6	1.002166 ± 0.000113

The numerical values of A_2 to A_7 will be found in Table 5.

Many of the solutions utilize one or more equations in common. It would obviously be impossible to derive more than six independent solutions given only the six independent equations. The reader should be warned that because these ten solutions are not observationally independent of each other it would be decidedly incorrect to try to arrive at the least-squares adjusted indirect value of Λ implied by the last six equations of Table 5 by applying the familiar weighted averaging method permissible for independent data (according to which the weights are made inversely proportional to the squares of the error measures) to the ten results of Table 6. The easiest way on the contrary is to perform a least squares adjustment of the last six equations of Table 5 since these equations have purposely been formulated so as to ensure their observational independence. E. R. Cohen has

devised a beautiful and simple way[26, 27] of finding the least squares adjusted *indirect* value of a quantity implied by an overdetermined set of equations, that is to say the least squares adjusted value implied by the entire set of input equations *except* the one which states the directly measured value. We use this method to obtain the least-squares adjusted indirect value of Λ.

In Figure 3, we show graphically, to very expanded scale, these ten solutions

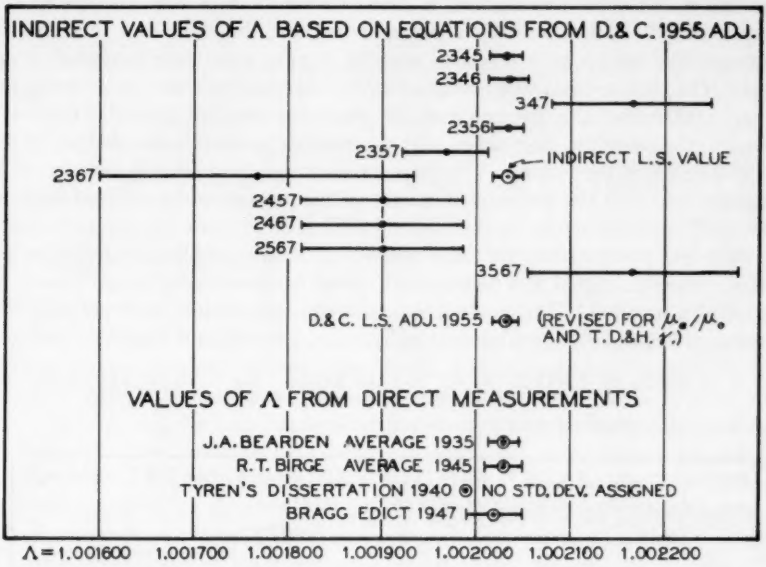

FIG. 3.—Ten different indirect solutions for the conversion constant, Λ, derived from the input data of the 1955 Least-Squares Adjustment of Cohen, Dumond, Layton, and Rollett are here graphically compared with the least-squares adjusted value for Λ of that adjustment and with various directly observed values. In this figure the input data of the adjustment correspond to the data of Table 5, i.e., they have been altered from the original values of 1955 to correct them for the Karplus & Kroll computational error of 14.3 ppm in μ_e/μ_0 discovered in 1958.

and their error measures together with the L. S. adjusted indirect value implied by all of them. In this same figure we also show the least-squares adjusted output value of Λ implied by the entire overdetermined set of equations of Table 5 including the directly measured information. It is worthy of note that the correction of the Karplus and Kroll value of μ_e/μ_0 has brought the L. S. adjusted value of Λ from 1.002039 ± 0.000014 (as given in 1955) down to 1.002031, in better agreement with the Bearden-Birge estimated average value from the direct measurements. We also show in Figure 3, for comparison, Bearden's and Birge's averages of the direct measurement, Tyrén's value (uncorrected for Lamb shift) and the Bragg edict value of 1947.

One of the important contributions to the data of Tables 5 and 6 is the fourth equation of Table 5 based on the well-known measurement in 1950, by Thomas, Driscoll, and Hipple, at the U.S. National Bureau of Standards, of the gyromagnetic ratio of the proton. The experiment was done in the strong field between the iron pole pieces of a powerful electromagnet.

The absolute measurement of the field in gauss was accomplished by weighing the force on a current-carrying wire placed in the magnet gap. The result obtained was, before correction for the diamagnetism of the sample,

$$\gamma_p' = \omega_p/B = (2.67523 \pm 0.00006) \times 10^4 \text{ radians sec}^{-1} \text{ gauss}^{-1} \text{ (T. D. \& H., 1950.)} \quad (23)$$

Now this ratio has been remeasured in 1958 under the U.S. N.B.S. auspices by P. L. Bender and R. L. Driscoll[28] by a different method, this time in a much weaker magnetic field set up by a precision solenoid of very accurately measured dimensions. The proton-containing sample (water) was first polarized in a strong field (about 5,000 gauss) at a distance from the apparatus and shot through a pneumatic tube into the energized solenoid so that its magnetic polarization ended up pointed along the axis of the latter. A short pulse (lasting about 10 milliseconds) of RF magnetic field near the resonant frequency of the protons in the solenoid field, was then applied perpendicular to the solenoid field so as to cause the magnetization of the sample to precess freely for many seconds in the solenoid field and this free precession induced a signal in a pick-up coil, whose frequency after amplification was accurately measured. The result of this measurement yielded a value of the proton gyromagnetic ratio 37.4 ppm lower than Thomas, Driscoll, and Hipple's.

$$\gamma_p' = (2.67513 \pm 0.00002) \times 10^4 \text{ radians gauss}^{-1} \text{ sec}^{-1} \text{ (B. \& D., 1958)} \quad (24)$$

The reason for this discrepancy is not yet clarified.

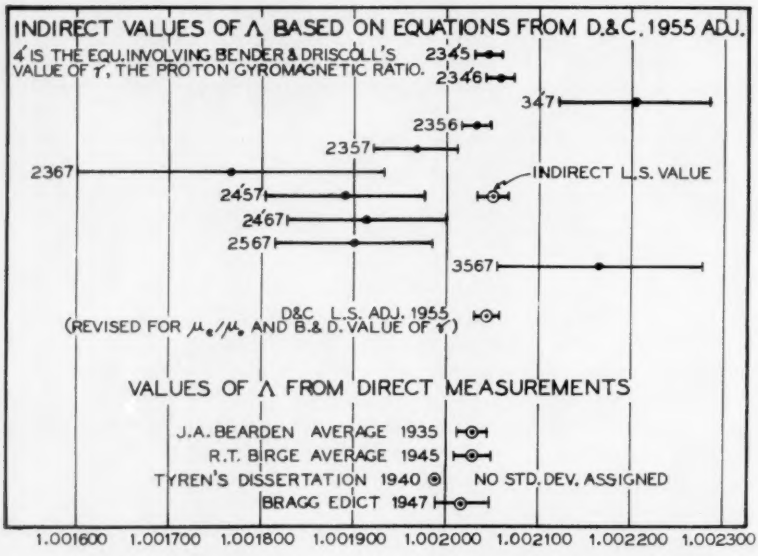

FIG. 4.—The ten different indirect solutions for the conversion constant, Λ, displayed in this figure differ from those in Figure 3 in that the constant, A_4, used in the computations has been changed from $A_4 = -3.7$ to $A_4' = -7.4$. This is the change required if we use Bender & Driscoll 1958 value of γ, the gyromagnetic ratio of the proton, to replace the Thomas, Driscoll, and Hipple 1950 value of that constant occurring in the fourth equation of Table 5.

If now we replace Thomas, Driscoll, and Hipple's γ with Bender and Driscoll's γ, we must change A_4 in the equations of Table 5 from $A_4 = -3.7$ to $A_4' = -7.4$. This will alter five of the solutions for Λ given in Table 6, namely those solutions involving A_4 and designated as 2345, 2346, 347, 2457, and 2467. Table 7 displays these five altered solutions and Figure 4 shows graphically all ten solutions for Λ including those altered by using the Bender and Driscoll datum to replace that of

TABLE 7

RECOMPUTATION OF FIVE OF TEN DIFFERENT JUST-DETERMINATE SOLUTIONS FOR Λ OF TABLE 6 WHICH REQUIRE MODIFICATION IF B. & D. VALUE OF γ REPLACES THE T. D. & H. VALUE

		Parts in 10^8	σ, ppm	Λ
(1)	$x_4 = {}^1/_3(A_2 + 3A_3 - A_4' - A_5)$ =	2.53	14.4	1.0020453 ± 0.000014
(2)	$x_4 = {}^1/_3(A_2 + 3A_3 - 2A_4' - A_6)$ =	3.73	14.8	1.0020573 ± 0.000015
(3)	$x_4 = 2A_3 - A_4' - A_7$ =	18.2	82.3	1.0022020 ± 0.000082
(7)	$x_4 = {}^1/_3(2A_2 + A_4' - 2A_5 + 3A_7)$ =	−13.1	85.8	1.0018890 ± 0.000086
(8)	$x_4 = {}^1/_3(2A_2 - A_4' - 2A_6 + 3A_7)$ =	−10.7	85.6	1.0019130 ± 0.000086

The numerical value of $A_4 = -3.7$ has been changed here to $A_4' = -7.4$ parts in 10^8 and its standard deviation has been changed to ±7.66 ppm (B. & D.'s value).

Thomas, Driscoll, and Hipple. The least squares adjusted value of Λ in Figure 4 has also been revised to take into account the change in the input value of γ.

Only three of the ten indirect solutions for Λ displayed either in Figures 3 or 4, namely 2345, 2346, and 2356, are sufficiently accurate to be of interest to us. When we compare the data displayed in the two figures it is clear that the use of the Bender and Driscoll data renders the set somewhat less consistent than does the use of the Thomas, Driscoll, and Hipple datum. It is of considerable interest that the solution, 2356, which does not involve the gyromagnetic ratio determination, A_4, yields a value of Λ which agrees quite well with both the L. S. adjusted value and with Birge's average of the directly measured values, whereas the solutions, 2345 and 2346, for Λ which do depend on A_4, are thrown from good agreement with the L. S. adjusted value and with Birge's average of the directly measured values into disagreement with these when the B. & D. datum replaces the T. D. & H. datum.[‖] Because of the uncertainty at the present moment as to which gyromagnetic ratio determination is the more reliable, the single indirect determination, 2356 acquires considerable importance as the only other source of information on Λ beside the directly measured values. We therefore select it as the one whose experimental bases we shall enumerate in detail to serve as example of the entire set of ten.

DATA OF SOLUTION 2356 FOR Λ

Linearized Form		Primitive Form		
$x_2 + 3x_4 = A_2$;	$N\Lambda^3$	$= c_2 = Mf/(\rho\phi d_x{}^3)$		(25)
$x_1 = A_3$;	α	$= c_3 = 4\Delta E_D{}^{1/2}[1 + ({}^5/_8)\alpha^2]^{-1/2}[2(\mu_e/\mu_0) - 1]^{-1/2}$ $R_D{}^{-1}c^{-1}$		(26)
$x_2 + x_5 = A_5$;	Ne	$= c_5 = cF_I$		(27)
$3x_1 + 2x_2 + x_3 = A_6$;	$Ne^2\alpha^{-3}$	$= c_6 = c^2(\mu_e/\mu_0)M_p/(4\pi\mu' KR_\infty)$		(28)

The numerical values associated with these quantities are:

Parts in 10^8	σ (ppm)	σ^2 (ppm)^2	
$A_2 = 3.5$	38.	1440.	$c_2 = 0.606179 \times 10^{24}$ mole^{-1} (Phys.)
$A_3 = 2.6$	4.5	20.2	$c_3 = 0.00729719$ (dimensionless)
$A_5 = 11.1$	13.1	172.	$c_5 = 2.89365 \times 10^{14}$ esu mole^{-1} (Phys.)
$A_6 = 14.9$	11.	121.	$c_6 = 3.57659 \times 10^{11}$ (esu)^2 mole^{-1} (Phys.)

Explanatory notes to above equations follow.

(25) The experimentally measured quantities in the right-hand member of this equation are the molecular weight, M, of a crystal; its macroscopic density, ρ; the geometric factor, ϕ, which differs from unity for rhombohedral crystals such as calcite as explained earlier in this paper; and the interplanar spacing, d_x, measured by means of X-rays and expressed therefore in X-units. All these quantities share in contributing to the error of the result. They appear in none of the other equations, however. f is a pure number, the number of molecules of molecular weight, M, per unit cell. The average value of $N\Lambda^3$ computed by Birge[29] from five different crystals was used.

(26) In this equation the experimentally measured quantities in the right-hand member are ΔE_D, the fine structure splitting in deuterium as measured by Dayhoff, Triebwasser, and Lamb;[30] the Rydberg constant for deuterium, R_D; the velocity of light, c; and the fine structure constant, $\alpha = 2\pi e^2/(hc)$. (It is permissible to use a less accurately known value of α as an input datum here because the bracket, $[1 + {}^5/_8)\alpha^2]^{-1/2}$, is very insensitive to small changes in α). For the anomaly ratio, μ_e/μ_0, of the magnetic moment of the electron to the Bohr magneton, the theoretical formula of quantum electrodynamics[31-34]

$$\mu_e/\mu_0 = 1 + \alpha/(2\pi) - 0.328\,\alpha^2/\pi^2 = 1.0011596 \qquad (29)$$

has been used. (The numerical value results from taking $\alpha^{-1} = 137.039$.) The only substantial contributor to the error in formula (26) is ΔE_D.

(27) In this equation the experimentally measured quantities are F_I, the Faraday constant as determined electrochemically by means of the iodine coloumeter and c, the velocity of light. The latter is needed as a conversion factor because, in the left-hand members of the equations, e denotes the electronic charge in absolute electrostatic units. Only the iodine Faraday is used because the equation corresponding to the Faraday by the silver voltameter was one of those rejected under strong suspicion of systematic error as described above. The discussion of this and other points too lengthy for inclusion in this paper together with descriptions of all the experiments here alluded to may be found in a book *Fundamental Constants of Physics*.[35] Only F_I contributes substantially to the error in this equation.

(28) In this equation the experimentally measured quantities in the right-hand member are c, the velocity of light; M_p, the mass of the proton on the "physical scale" of atomic masses; μ', the magnetic moment of the proton expressed in nuclear magnetons, $\mu_n = eh/(4\pi m_p c)$ (here m_p is the absolute mass of the proton), as determined by Sommer, Thomas, and Hipple;[36] $K = (\mu_e/\mu_p) = [M_p/Nm\mu'] \cdot (\mu_e/\mu_0)$ is the measured ratio of the electron magnetic moment, μ_e, to the proton magnetic moment, μ_p, as measured by Koenig, Kusch, and Prodell[37] and R_∞ is the Rydberg constant for infinite mass. Here μ' is the only substantial error contributor, all the other quantities being treated as fixed auxiliary constants including μ_e/μ_0 which as explained under (26) is computed from the theoretical formula.[¶]

The formula, 2356, written first in its linearized form and then in its primitive form reads:

$$x_4 = (1/3)(A_2 + 3A_3 - 2A_5 + A_6); \qquad (30)$$

$$\Lambda = (c_2 c_5^3 c_6/c_5^2)^{1/3} = [(N\Lambda^3)\alpha^3(Ne^2/\alpha^3)/(Ne)^2]^{1/3} \qquad (31)$$

The last equality in (31) is easily verified.

What we have done in this indirect solution then can be described in words as follows. We use the value of the Sommerfeld fine structure constant, α, as determined by Dayhoff, Triebwasser, and Lamb's measurement[30] on deuterium along with the determination of Ne^2/α^3 (computed from the Sommer, Thomas, and Hipple "omegatron" measurement of the magnetic moment of the proton[36] combined with five other very accurately measured constants as described under (28) to obtain a value of Ne^2. The square of the electrochemically measured Faraday constant gives us a value of N^2e^2. The quotient then of this latter by Ne^2 gives us N, the Avogadro number. The measurements[29] based on the densities, geometry, molecular weights, and grating constants of five different crystals (the latter determined by X-rays on the X-unit or Siegbahn "crystal scale") gives us the product, $N(\lambda_g/\lambda_s)$ and the desired quantity, λ_g/λ_s, is simply the quotient of $N(\lambda_g/\lambda_s)$ by the preceding value of N.

It should be clear then from this and from the discussion of the measured quantities in equations (25)–(28) what a wide variety of precise physical measurements from many branches of physics and physical chemistry are involved in this indirect determination of Λ, a determination whose result nevertheless agrees quite satisfactorily with Birge's average of the direct measurements of Λ. The two approaches are totally independent.

The indirect solution for λ_g/λ_s which we have just described is only one out of ten different indirect solutions permitted by the 1955 data and involving a wide variety of sources drawn from almost every field of physics. The close and intricate linkage between precision X-ray measurements and all the rest of physics is here well illustrated and indeed it is becoming increasingly clear to nuclear physicists,[40] solid state physicists,[41] and even to physicists studying mesons[42] and other strange particles that they cannot do without precise data from the field of X-rays, data expressed in terms whose definitions must be clear, precise, and unambiguously related to the entirety of the physical system of units and measurements if we are to make progress. It is our duty to see that this comes to pass. Adoption of an arbitrarily defined value for λ_g/λ_s unrelated to the rest of physics is therefore not a step in the direction of progress.

Conclusions as to Our Present Knowledge of Λ and Their Implications for Tabulating X-Ray Wavelengths in Milliangstrom Units.—It seems fairly clear from the analysis of the data summarized in Figures 3 and 4 that the value of Λ indicated by our present sources of knowledge must be not far from

$$\Lambda = 1.002031 \pm 0.000014 \text{ ('55—L. S. adjusted value corrected for } \mu_e/\mu_0). \quad (32)$$

This is the least-squares adjusted value implied by all seven equations of 1955, including the direct as well as indirect data and using *Thomas, Driscoll, and Hipple's γ, after those equations, have been corrected for the error discovered in 1948 in the Karplus & Kroll computed value of μ_e/μ_0.* The as-yet unresolved dilemma between the Thomas, Driscoll, and Hipple-γ and the Bender and Driscoll-γ makes it necessary to take into account also the least-squares adjusted value of Λ implied by the seven equations, direct and indirect, of 1955 with the correction for the error in μ_e/μ_0 *and with the Bender and Driscoll γ.*

$$\Lambda = 1.002044 \pm 0.000014 \text{ ('55—L. S. adjusted value corrected for } \mu_e/\mu_0 \text{ with B\&D-}\gamma) \quad (33)$$

The discrepancy between T. D. & H. and B. & D. is as yet unexplained.** Because of this uncertainty we have at the present moment no choice but to take as our present best information on Λ, an equally weighted mean between the two results given in (32) and (33) which, fortunately, do not differ significantly compared to their standard deviations. It would be quite incorrect to compute the error measure in this mean, however, as though the two results (32) and (33) were independent since they are in fact strongly correlated. The writer suggests the value

$$\Lambda = 1.002037 \pm 0.000020 \text{ (DuMond's best guess, 1959)} \tag{34}$$

The error measure, it will be noted, has been expanded a little "for safety," to cover "possible but unknown" systematic errors. (This sort of guesswork is a prevalent custom which the writer deplores, *especially when it is done without disclosure.* In the present case the reader is at least warned.)

It is fairly evident then that we cannot at present regard Λ as known with a standard deviation much smaller than 20 parts per million. Whether the efforts now in progress to improve upon this precision will effect an improvement of a whole order of magnitude seems doubtful at the present time. For example, the grazing angle of diffraction of the silver $L\alpha_1$ X-ray line diffracted from a grating of 600 lines per mm under a grazing incidence angle of say 10 minutes is 1.29 degrees in the first order and 3.14 degrees in the 6th order. Because the wavelength, λ, is about proportional to the square of the angle of diffraction, in order to measure λ to ±20 ppm the angle must be measured to ±10 ppm. Hence in the more favorable of the two above cases (the case of the 6th order) the diffraction angle must be measured to ±0.11 seconds of arc to match the precision with which we now believe we know Λ. To improve on this precision by a whole order of magnitude, much as we would like to do it, promises to present serious difficulties, whether we hope to do it either by the method of comparison with hydrogenic spark lines or by absolute angle measurements.

Now let us turn to the accuracy with which X-ray wavelengths can be measured on the crystal scale. In the most recent wavelength tables given by A. E. Sandström in the *Handbuch der Physik* these are frequently quoted to six and in many cases to seven significant figures. Over 70 reference lines are there tabulated in X-units out to the third decimal place of that unit. From the writer's own experience with the two-crystal spectrometer this precision is perhaps about the limit of what can be done by very careful replicated work with a precision instrument capable of measuring angles to a small fraction of a second of arc, say 0.1 second or smaller. Taking as a typical case the wavelengths of the $MoK\alpha_1$ line this precision corresponds to about 1.3 parts per million. It should be clear then that a sacrifice of at least a factor of ten or twenty in precision will be the price paid for expressing all these lines on a scale of milliangstroms.

Desirability of Redefining the X-Unit in Terms of One or More Standard X-Ray Emission Lines.—At present most textbooks refer to the "crystal scale" of x-ray wavelengths and define the "X-unit" as a unit such that the "effective" value in first order reflection of the grating constant of the cleavage planes of "purest" or "perfect" calcite at 18°C is

$$d_{18}'' = 3029.040 \text{ X-units.} \tag{35}$$

Different samples of calcite from different parts of the world have been shown by J. A. Bearden[38] to reflect one and the same X-ray line at slightly different Bragg angles ranging over a variation in the first order of some 0.3 second of arc to 0.7 second of arc in the fourth order. Similar small variations have been observed by K. Lonsdale for apparently perfect specimens of diamond. But how then does one know when he is using a sample of "purest" or "perfect" calcite? The answer is that in practice one standardizes one's crystal by measuring a standard oft-measured X-ray line with it such, for example, as the MoKα_1 line. Our practice of defining the unit with which X-ray wavelengths can be most precisely measured in terms of a crystal grating space is simply a vestige of the historical fact that at the outset first Sir William Bragg, then A. H. Compton, and finally Manne Siegbahn based their computation of these wavelengths upon the density, geometry, and molecular weight of a calcite crystal. If, however, calcite were strictly adopted as the conventional standard to define the X-unit it would thenceforth be strictly necessary for the International Bureau of Weights and Measures at Sévres to keep in a desiccator, under extremely carefully controlled conditions, one standard calcite crystal, or perhaps a pair of them for use in a two-crystal spectrometer, against which all other crystals for use by X-ray spectroscopists could be compared. Such a cumbersome procedure is clearly not in the spirit of modern physics where every effort is made to relate our standards to the most fundamentally reproducible natural units or constants obtainable. Just as the meter is, for the most accurate purposes, defined in terms of the wavelength of a spectral line in the optical region, so the x-unit also should be defined in terms of one or more X-ray lines, simply because this is the most reproducible and accurate way to define it. We do not imply by this proposal a *shift in the value of the x-unit*, merely a *sharpening* in the precision of its definition. For this purpose we must select a standard X-ray line or lines whose wavelengths can readily and precisely be compared, by crystal diffraction or other methods, with the wavelengths of most of the several thousand other known X-ray lines (using single or successive multiple steps of comparison with one or more different crystals as tools). Perhaps even some appropriately weighted average of a multiplicity of standard lines, whose wavelengths relative to each other have been determined with exceeding care should be adopted (see an appendix to this paper) as a sort of *collective standard*. In all such work the various crystal grating constants are to be regarded as merely the tools of comparison but the lines are the fixed points in the scale. At the present moment a proposal is extant, with the signatures of Manne Siegbahn and other X-ray people, to define the X-unit at a specific numerical value in terms of the wavelength of the MoKα_1 line. The writer is strongly in favor of adoption of such an improvement in the sharpness of what is meant by the term x-unit, since it would be a distinct step in the direction we should go. The writer does not urge the particular choice of MoKα_1 as a point of great importance. Another choice such as CuKα_1 might turn out to be better and more convenient or again a collective unit based on a weighted average of a multiplicity of very carefully measured standard lines might be even more to be preferred. The important point emphasized here is the adoption of emission lines as the fixed points to define the scale in preference to crystals.

What Feature of an X-Ray Line Profile Shall Be Taken as "The" Wavelength?—The two-crystal spectrometer affords a spectral resolving power amply sufficient

to reveal the "natural structure" of the spectral profile of an x-ray emission line with considerable fidelity. For example, in the case of first order reflection of the tungsten $K\alpha_1$ line from the (310) planes of quartz, the planes lying normal to a 1-mm. thick slab of crystal with the beam transmitted through the slab, a parallel position rocking curve of half width at half maximum height of about 1.4 seconds of arc was obtained (an index of the order of angular and spectral resolving power of the crystals) and this is to be compared with a half width at half maximum height of 14 seconds for the natural line profile,[39] obtained with the same crystals. Unfortunately there is no unanimity of convention as to what feature of the spectral profile of an X-ray line to take as "the" wavelength. The maximum point, the center of gravity or "centroid," the point of intersection of the tangents to the two points of inflexion of the profile, the mean position of the median points of horizontal chords (after subtraction of the background) and the limit of the locus of such median points extended to the peak of the line, have all been used or proposed. In the case of nearly all the X-ray lines determined with photographic spectrometers, a cross hair in the microscope of a comparator is simply made, by a subjective judgment, to divide the blackened image as nearly in its middle as can be estimated. We believe that this latter process comes nearer to determining the median point of horizontal chords near the peak of the profile than to any of the other procedures.

When the natural line profile is distorted (as it must necessarily always be to some extent) by various instrumental causes and other experimental impurities it is sometimes necessary to resort to various "folding" and "unfolding" procedures to reduce one's data. Now there is a beautiful and simple additive theorem relating the centroid of the fold of two profiles into each other to the centroids of the two individual profiles which makes the adoption of the centroid as the fiducial feature of a line profile very attractive and convenient.

Unfortunately there is a real difficulty with adopting the centroid of the profile as the fiducial feature in the case of X-ray lines. It is well known that the "witch" or Cauchy distribution,

$$I(\lambda) = I_{\max} [1 + (\lambda - \lambda_0)^2/W^2]^{-1} \tag{36}$$

sometimes also called a "Lorentzian," describes to quite a good approximation the natural spectral profile of many X-ray lines and in particular does so rather well out to several half widths, W, as regards the law of decay of the "tails" of the line, a law which at great distances is like the inverse square of the distance from the peak. In a strict mathematical sense the witch profile has no centroid since its first moment diverges logarithmically. It is, of course, an easy matter to redefine what we mean by "the centroid," provided the profile is strictly a Cauchy distribution, or indeed simply provided the profile possesses an intrinsic axis of symmetry, so as to establish the center of symmetry in a definite way. But to do this we must always "beg the question" a little by making appeal to some other feature of the profile beside the centroid in order, by successive approximations to ensure that the limits over which we extend our integral in determining the centroid, shall be sufficiently symmetrically located relative to the peak. But if the profile has no intrinsic axis of symmetry, i.e., if the curve is not an even function but a sum of an even and an odd function, then the slow decay of the tails of the lines can introduce

a considerable shift in the position of the centroid and indeed one cannot be strictly sure about this position without exploring the tails of the curve out to very great and unfortunately quite indeterminate distances from the peak. Now the unfortunate truth is that most X-ray lines when studied with great care are not strictly Cauchy-like in shape and worst of all *do not have an axis of symmetry* but they do exhibit the slow law of decay (approximately inverse square) in the tails which is the root of all this trouble since it renders the position of the centroid objectionably sensitive to small vagaries and asymmetries of profile at large distances. In practice we do not know where to chop off the tails of the line in choosing the beginning and ending points of our integration to determine the centroid. If we truncate the tails too much and the line has a small unsuspected asymmetry extending far into the tails we may be incorrectly biasing the centroid position by our arbitrary choice of the truncation points. If, on the other hand, we extend our integration limits too widely we shall be introducing unnecessary uncertainty into the centroid position because of the random statistical fluctuations in the tails, an uncertainty which increases as the square root of the distance we extend the limits. For these reasons the centroid position is perhaps the poorest feature of an X-ray line profile from the point of view of definiteness and reproducibility.

There is a still more important consideration in this connection, however (than the uncertainties attending the determination of the centroid), as a warning against its indiscriminate or incautious use. This is the fact that the present tabulated X-ray wavelengths *do not refer to the centroids of the corresponding line profiles.* The great majority of these wavelengths have been photographically determined by placing the cross hair of a comparator microscope subjectively on the center of a streak of photographic blackening and they refer to something that may be described roughly as the peak and which this writer believes is probably best found by the procedure above referred to as the limit of the locus of the centers of chords extended upward to the peak of the profile. It is possible that, with a great deal of careful labor exploring profiles with the two-crystal spectrometer out to many half-widths, the shift of the centroid of any specified line profile away from the peak position (as defined above) could be determined. Frequently quite closely adjacent lines in the spectrum have different asymmetries so that the relationship between centroid and peak is today an unknown quantity which would have to be determined for a very great number of lines. It is therefore problematical whether the mathematical simplification gained by use of the centroid will make this worth the effort in view of the real experimental difficulties in fixing the centroid by reason of the extended asymmetries and statistical fluctuations in the tails aforementioned.

SUMMARY

The system of some 3,000 X-ray emission line wavelengths, some of which have been (and many more of which can be) measured *relative to each other* to a precision approaching a very few parts per million by refined crystal diffraction methods, constitutes an extremely reliable and valuable set of fixed points in our scale of length magnitudes in the general region of atomic dimensions. Manne Siegbahn, circa 1930, utilizing the best values then available of N, the Avogadro number, combined with the density of calcite, the geometry of its lattice and the atomic weights of its constituents, computed a value for the grating spacing of the

cleavage planes of calcite. After taking into account the effect of the refractive index of X-rays in calcite this led to a value for the "effective" grating constant for first order Bragg reflection from the cleavage planes of 3,029.040 milliangstroms. This value was incorrect because the value of N which he had used in its computation was erroneous, but Siegbahn, who had no way of knowing this, used this erroneous result to establish a measuring unit for X-ray wavelengths which he intended to be 1 milliangstrom (10^{-11} cm) but which he very wisely called by a distinctive name "the x-unit" probably because, even at that time, he felt less security regarding his knowledge of its relation to our macroscopic standards than he could wish in view of the excellent reproducibility of the X-ray wavelength measurements relative to each other. For this purpose Siegbahn had taken a value of N computed as the quotient of the Faraday by Millikans "oil drop" value of the electronic charge. This last has later been shown to be too small by nearly 0.6 per cent. More precise later direct measurements of X-ray wavelengths using artificial ruled gratings have shown that the total effect of all the systematic errors in the constants Siegbahn adopted made his X-unit a little over twenty parts in ten thousand larger than one milliangstrom. Since the chief uncertainty regarding the absolute value of X-ray wavelengths concerns only the value of a single conversion factor, $\Lambda = \lambda_g/\lambda_s$, i.e., the ratio of a given wavelength expressed in milliangstroms, λ_g (on the "grating scale"), to the same wavelength expressed in X-units, λ_s (on the Siegbahn or "crystal" scale), it has become customary, in quoting X-ray wavelengths to high precision, to express them on Siegbahn's nominal scale of X-units defined in terms of the calcite grating space. Calcite, however, has been shown to be less reproducible from sample to sample than are the wavelengths of appropriately selected X-ray lines so that for purposes of maximum precision and reproducibility, this author believes it would be still better to define the X-unit (or some arbitrary unit for measuring X-ray wavelengths) in terms of one or more carefully selected X-ray emission line wavelengths.

This paper surveys all our present sources of both direct and indirect information on the conversion constant, Λ, and examines their probable reliability. Such a survey is of current interest because of a proposal now seriously being discussed among X-ray crystallographers to express all the standard X-ray emission line wavelengths, whose values are to be published in the new International Tables of Crystallography, in angstrom units. It is alleged that this step should be taken in the interest of simplification and to avoid errors and misunderstandings. This paper aims to show that unless the precision of our present knowledge of Λ can be improved by at least an order of magnitude, the above proposal will require either (1) seriously downgrading the precision with which all the emission line wavelengths are quoted (in angstrom units) for the use of crystallographers or (2) adopting some *conventionally defined value* of the conversion constant, Λ. In the latter case the numerical values of the X-ray wavelengths arrived at by its use will be neither fish nor fowl for they will be expressed neither in x-units nor truly in angstrom units but in a third new conventional unit which, unless it be given a distinguishing name, will lead to even more confusion than at present. Emphasis is placed on the fact, frequently overlooked by specialists in a given discipline, that the entire system of measurements of Physics and Chemistry has become so closely and quantitatively interrelated (an interrelationship which becomes ever tighter and more intricate

with time), that we cannot arbitrarily tamper with one part of the system without affecting all the rest. The temptation to dry to circumvent a metrologically difficult determination of a badly needed conversion factor by adopting a conventional definition for it worsens the situation by introducing overdetermination, inconsistency, and semantic confusion. Ten different "indirect" solutions for Λ, computed by appropriately combining different precise measurements of combinations of the atomic constants (other than the direct determination of X-ray wavelengths by means of gratings) are given. Two least-squares adjusted indirect values implied by all this information area:

$$\Lambda'_{(\text{Indirect})} = 1.002034 \pm 0.000016 \text{ (using T. D. \& H value of gamma)}$$

$$\Lambda''_{(\text{Indirect})} = 1.002051 \pm 0.000016 \text{ (using B. \& D. value of gamma)}$$

depending, respectively, on whether the value of gamma, the proton gyromagnetic ratio as measured by Thomas, Driscoll, and Hipple, or that of Bender and Driscoll is used. (In these indirect calculations only the iodine value of the Faraday has been used. See footnote below with symbol‖).

These values are to be compared with the average of directly measured values of Λ of Birge (and also earlier of J. A. Bearden) namely

$$\Lambda_{(\text{Direct})} = 1.002030 \pm 0.000020 \text{ (Birge, Bearden).}$$

The direct and indirect values depend on completely independent sources of data Since the discrepancy between these two values of gamma (T. D. & H. or B. & D) remains so far unexplained we have no choice for the moment but to compute Λ under both hypotheses and take the average. The least-squares adjusted best value from both indirect and direct sources of information taking an equally weighted average between the results obtained when the two different values of gamma are used, yields

$$\Lambda = 1.002037 \pm 0.000020 \text{ (DuMond's best guess, 1959).}$$

The error estimate here has been slightly increased above the calculated standard deviation (±0.000014) to allow for the possibility of systematic errors.

Summary of Conclusions.—It is the present opinion of the writer that:

1. The conversion constant, $\Lambda = \lambda_g/\lambda_s$, is not at present known with sufficient precision to permit tabulating the standard x-ray emission line wavelengths in milliangstrom units without a serious sacrifice of the precision with which these wavelengths are now known relative to each other. Since X-rays are an important part of the general structure of Physics it is undesirable to adopt a conventional value for Λ which may later turn out to be inconsistent with the remainder of that discipline.

2. The unit in which X-ray wavelengths, using crystal diffraction methods, can be measured with highest accuracy should be redefined by tying it to one or more standard X-ray emission lines and abandoning its present conventional definition in terms of "purest" or "perfect" calcite.

3. Great care and circumspection should be used in defining the fiducial reference point on the natural profile of an X-ray line which shall be taken as "the wavelength." The tabulated wavelengths at present in general usually correspond most nearly to the peak because of the methods used in their determination.

The centroid of the profile does not necessarily coincide with the peak by any means and while it possesses certain attractive mathematical properties the difficulties of establishing its relationship to the tabulated peak values in a reliable way are considerable and as yet not in most cases surmounted.

APPENDIX

A Tentatively Suggested Method of Defining a Collective X-Ray Standard of Wavelength Based on a Multiplicity of Selected and Very Carefully Measured Emission Lines.—The suggestion in the body of this paper that a precision unit for measuring X-ray wavelengths could be defined in terms of a multiplicity of selected standard emission lines, perhaps requires a little amplification to make the contemplated procedure clear. It has been pointed out in correspondence by M. Siegbahn, Y. Cauchois, and others that probably no single reference line can be chosen which will serve equally well as a standard for work in all regions of the extensive X-ray spectrum. The spectral gamut of γ-rays and X-rays which has been studied by crystal diffraction is so huge (extending from 6.2 X-units wavelength in the case of some of the studies of neutron-capture gamma radiation from titanium by J. W. Knowles at Chalk River, Canada, to 10,000 X-units in the case of very soft X-rays) that crystals of different kinds and with different interplanar spacings are naturally chosen as most suitable for studies in different regions. This does not, of course, preclude setting up a reliable precision scale over the entire gamut because there is a wide variety of good crystals from which to choose and each crystal is suitable for work over such a wide spectral range that these different ranges overlap very generously and afford ample opportunity to measure numerous X-ray lines in common when the different interplanar spacings of different crystals are being precisely intercompared. It would, however, be clearly much more convenient to have a multiplicity of reference points in the X-ray wavelength scale distributed in such a way that one or more of them would be readily and conveniently available for standardizing almost any crystal or any general spectral domain with which an investigation might be concerned. Naturally this entire system of standard reference lines should have had the wavelengths of its members all measured and intercompared with extreme care, and by different laboratories, using as many varieties of crystal as needed to obtain best results. When sufficiently satisfactory agreement as to the relative wavelengths of these reference lines would be reached it should be possible, in terms of the statistical uncertainties of the mean values of each of the measured standard wavelengths, to attach weights (inversely proportional to the squares of said uncertainties) to each reference line wavelength. Then the X-unit (if that name may still be retained) would be defined by giving the following procedure for determining to high precision the "effective" d in X-units for any reflection in any crystal one might be planning to use. One would measure, with the crystal to be normalized or standardized, the "effective" d as indicated by the Bragg angles observed with as many of the standard reference lines as were conveniently accessible with that crystal. Because of small errors of measurement each reference line would yield a slightly different d for the crystal planes in question. The weighted average of all these d's, using the standard weights already established for the different reference lines, would then be taken as the d of that set of crystal planes in that crystal. This procedure has the advantage that it would permit

the worker standardizing his crystal to choose, from a wide range of possibilities, any degree of precision he might desire consistent with his requirements. To standardize a given set of crystal planes with the highest possible precision he would measure the largest possible number of reference lines accessible to that crystal which his facilities would permit. The weighted average of the d-values obtained with all of these lines would then give the crystal's d as accurately as it could be ascertained. Its accuracy could furthermore be reliably estimated by the familiar methods of computing the precision measure of the mean of weighted observations. On the other hand a standardization of this crystal in terms of one single most convenient reference line might yield sufficient accuracy for the purposes contemplated, in which case the other reference lines would be ignored. Thus the d of a crystal standardized in terms of nine reference lines whose wavelengths might have been preestablished say with equal precision would be known three times as accurately as one which had been standardized with only one of the lines. The collective X-unit defined in this way by the entire array of reference lines would be more accurately fixed than it could be defined in terms of any single line.

Since X-rays comprise a very important range of the physical quantum energy spectrum, and since this is intricately and inextricably involved with all the rest of the measurements made by physicists in every branch of their all-important discipline, the duty devolves upon us to see that the highest possible precision is established here. In the long run nothing save the very best that can be done will be good enough.

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† These values are not the present international atomic weights but rather essentially the values used by Bragg in 1914 (with slight modification by Siegbahn[1] to obtain an accuracy of one additional significant figure). The present weight for rock salt is 58.454.

‡ Stenström in 1919 demonstrated the existence of a slight deviation from Bragg's law, $n\lambda = 2d \sin \theta$, which he correctly attributed to the fact that X-rays have an index of refraction in the material of the crystal slightly lower than unity. In order to satisfy Bragg's law connecting λ and θ, slightly different "effective" values of the grating constant, d, depending on the order number, n, must replace the true d. For high orders d-effective approaches the true d. In the case of Bragg reflection in the nth order from the calcite cleavage planes, an empirical formula for the effective grating constant d_n, is $d_n = [1 - 135 \times 10^{-6}/n^2]d$ where d is the true grating constant of those planes. This correction, however, only applies in this form to "Bragg reflection" where the X-rays enter and leave the crystal through the same boundary surface and the reflection is from planes parallel to that surface. For internal reflection in a slab of crystal from planes normal to the two (parallel) exit and entry surfaces the correction vanishes.

§ R. T. Birge has assured the writer that he had no part in deciding the manner in which this value was recommended to the public, a manner very foreign to Birge's own meticulous and detailed disclosures of his sources of information and bases for arriving at values.

‖ The writer is indebted to P. L. Bender who read the manuscript for pointing out the following remark in a private communication received after this manuscript had been submitted for publication. Quoting Bender directly: "In your evaluation of the X-ray conversion constant with our recent value for the proton gyromagnetic ratio, you have used only the iodine value for the chemical Faraday. While this experiment is perhaps less suspectible to systematic errors than the silver Faraday, I don't believe that you would have excluded the silver value completely in 1955 except for its conflict with the physical Faraday determined from the gyromagnetic ratio and omegatron experiments. The conflict, of course, came out in your treatment as a large value for chi-squared when the silver value is included. However, as we have pointed out previously, if our recent gyromagnetic ratio value is used with the omegatron result the physical Faraday is

about half-way between the iodine and silver values. It thus seems that for consistency one would have to include both values with some form of weighting. Clearly, a wide difference of opinion can occur as to what weightings should be used for the Faraday values and the gyromagnetic ratio results. This range of opinion seems to me to be the actual limit on our knowledge of the constants at present rather than the statistical errors quoted for the individual results going into the least squares adjustment." (End of quote.)

There is no doubt that had the silver Faraday been included among the data and given weight comparable to the iodine Faraday, the data using Bender's value of gamma would have appeared more consistent. However, the present writer's misgivings regarding the correctness of the silver Faraday are of much longer standing than the conflict with the "physical Faraday" referred to in the above quotation. They date in fact all the way back to 1940 when the writer noticed that the apparent difference between the two sets of values of e/m which R. T. Birge once distinguished as "spectroscopic" and "deflection" values could be largely wiped out by using the iodine Faraday to deduce the "spectroscopic" values in place of the silver Faraday. It then occurred to the writer that the fact that iodine is monoisotopic whereas silver occurs naturally in two isotopes of nearly equal abundance might be significant and he has been agitating ever since for a reinvestigation of this whole question in the light of our modern knowledge of isotopes. All the early precision determinations of the Faraday, circa 1900–1910, were made before the existence of isotopes and their selective participation in electrolysis was known. The question of the reliability of the silver Faraday is still an open one.

¶ It will be noted that each equation involves only one experimental factor which contributes significantly to the numerical error or uncertainty and a *different one* for each equation, all the other data in the equation being so much more accurately known as to contribute essentially not at all to the error. Care has been taken purposely to formulate the observational equations of the least-squares adjustment in this way for unless this is done, i.e., if two or more equations have an essential error contributing factor in common, the equations are not statistically independent, in which case the simple classical weighting procedure for finding the least-squares solution is incorrect. If two or more of the observational equations have an essential error contributing factor in common, it is in fact no longer possible to assign independent weights to the equations, but a weight matrix for the complete set must be used and the computation is considerably complicated thereby.

** McNish at the U. S. National Bureau of Standards has suggested that perhaps the discrepancy in the γ-results of T. D. & H. and B. & D. could be explained by the possible existence of a slight misalignment of the centers of the two circular iron pole faces of the T. D. & H. electromagnet. Apparently these faces were carefully checked for parallelism but not for centering on the same axis. Such a displacement could occasion a slight obliquity of the magnetic field from the normal to the faces (the direction it was assumed to have had) and this in turn would introduce a cosine error into the absolute measurement of the field intensity by the force on the current-carrying conductor. The proton resonance frequency, on the other hand, always corresponds to the absolute value of the field intensity independent of its direction. Unfortunately the magnet having been dismantled, it is now impossible to verify whether such a hypothetical misalignment of the poles actually existed in sufficient amount to explain the discrepancy. Unsuspected nonlinear effects in the relation between ω_p and B might also be present in either of the two experiments.

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*LASTING AFTER-EFFECTS PRODUCED IN RATS BY SEVERAL COMMONLY USED DRUGS AND HORMONES**

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This is an era of the widespread use of drugs and hormones in the treatment of a great variety of illnesses. New medications appear on the market almost every day; as a matter of fact, many of the members of this audience undoubtedly have been treated with one or more of these new therapeutic agents.

Although the use of many of these drugs has resulted in great gains in the treatment of various ailments, these highly desirable results have often been accompanied by the development during treatment of a variety of undesirable side-effects. In many instances these accompanying effects have been sufficiently serious to necessitate discontinuation of the medication. It is on this account that new drugs and hormones have for some time been screened using animals not only for possible immediate toxic effects but also for any side-effects that might develop during prolonged treatment.

The possibility that in addition to such concomitant side-effects the administration of some of the commonly used drugs and hormones may be followed by serious and permanent pathological after-effects has not received much, if any, attention.

I bring this up because we have noted in experiments made on rats for quite different purposes[1] that the prolonged administration of a number of commonly used therapeutic drugs and hormones has resulted in pathological effects that only appeared well after the end of the treatment; characteristically, once developed, these persisted throughout the remaining life of the animal. Though these effects revealed themselves both in behavior and in metabolism they were not obvious on ordinary inspection. Thus, the rats appeared to be quite normal—in particular, they were free of all the common signs of deficiency such as loss of hair, motor disturbances and nervousness. That despite this they actually were quite abnormal could only be detected through scrutiny of daily records, taken over long periods of time, of their spontaneous running activity in revolving drums, their food and water intake, and the functioning of their reproductive tracts.

Before presenting some records typical of these animals, a short description should be given of the conditions under which the observations were made. The rats were kept separately in activity cages consisting of a revolving drum, a cyclometer, and a small living compartment with a nonspillable food cup and a 100-ml graduated inverted water bottle.[2] Records were made daily of running activity, food and water intake, as well as of the vaginal smears. The external conditions, room temperature, etc., were kept as nearly constant as possible, and were free from any regularly recurring disturbances or variations.

Daily records were taken on each rat for several weeks or months before the start of an experiment. During the treatment period the medication was thoroughly mixed with the food, thus the animals ingested small amounts at each feeding period, that is, at 3–4 hour intervals throughout the 24 hours of each day. After the end of the treatment, daily records were continued either until it was certain that no demonstrable changes had occurred or, in cases where definite changes did occur, until the death of the animal, either by natural means or through its sacrifice.

The drugs and hormones used in these experiments are listed in Table 1, which also shows the number of rats given each medication, the ranges of: ages at the start of treatment, concentrations of the medications in the stock diet, lengths of the treatment periods, as well as lengths of the observation periods after the end of the treatment. Six drugs and three hormones, and a total of 57 female rats—most of them under 85 days of age at the start of the treatment period—were used in these experiments.

It must be made clear here that this was not a planned experiment: the observations were made incidentally in the course of a number of other experiments and were entirely unanticipated. This explains the wide range in the number of rats used with each of the various drugs and hormones, as well as variations in the lengths of the treatment and observation periods.

TABLE 1

		Number of Rats	Age at Start of Treatment in Days	Concentrations in Food Percent	Duration of Treatment in Days	Observation Period After End of Treatment in Days	Number of Rats With Abnormal Cycles	Percent of Rats With Abnormal Cycles
Antibiotic	Sulfamerazine	25	59–503	0.5–5	74–116	55–630	5	20.0
Anti-thyroids	Thiourea	3	52–60	1–3	85	84–398	1	33.3
	Thiouracil	5	51–85	0.2–1	44–92	280–554	4	80.0
	Propylthiouracil	7	61–67	0.1–1	157	80–244	3	42.8
Analgesic	Aminopyrine	6	53	1–2	116	70–770	1	16.6
Sedative	Sodium Barbital	4	65–75	0.1–1	124	232–258	1	25.0
Adrenal Hormone	Cortisone	3	52–64	0.01–0.04	105	46–240	1	33.3
Female Hormones	Estradiol	3	81	0.0001–0.001	141	375–655	2	66.6
	Progesterone	1	110	0.025 –0.15	492	239	1	(100)
TOTAL		57					19	33.3

These drugs and hormones were kindly supplied by Merck Sharp and Dohme; Ciba Pharmaceutical Products, Inc.; Schering Corporation; Lederle Laboratories Division; and the Sterling-Winthrop Research Institute.

For each animal, the experimental substance usually was initially offered in a minimal concentration, well below the toxic level. In some instances the rats received only this concentration. Thus 8 of the 25 rats on sulfamerazine received only the 0.5 per cent mixture. In other instances the concentration of the test substance was increased by small steps at intervals of several weeks or months until food intake showed just a small decrease. Then the drug was either discontinued after a short time or reduced to a lower level at once. Spontaneous activity cannot be used as an indicator of toxic effects since in some instances it is depressed by only small amounts of drugs or hormones.

Daily records from several thousand untreated normal females served as controls. From these records it is known that throughout most of its life, the healthy female rat shows regular peaks of running activity every 4 or 5 days—always coinciding with ovulation.[3] It is further known that the average daily activity of each rat remains fairly constant with the exception of regularly recurring variations associated with the estrous cycle. Moreover, in most instances, the average daily food and water intakes likewise remain fairly constant, i.e., free even from variations associated with the estrous cycle. Figure 1 gives a typical record of daily spontaneous running activity, food intake and vaginal smears, as well as weekly readings of body weight, for a normal female rat that was started in the cage at an age of 42 days. During the first 30 days in the cage, daily spontaneous running

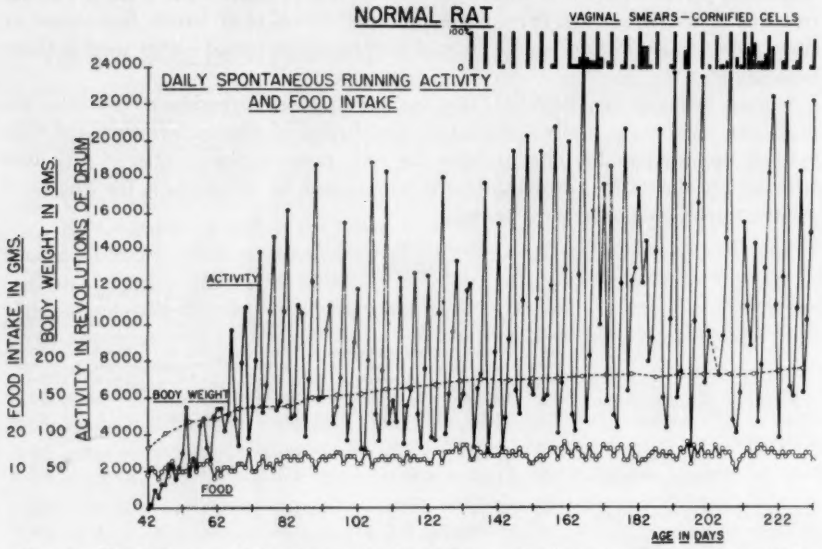

FIG. 1.—Graph for normal female rat showing daily record of spontaneous running activity, food intake, and vaginal smears, and weekly readings of body weight. Record shows the 4-day estrous peaks of activity.

activity increased from zero to more than 9,000 revolutions; food intake showed a slight increase; while during the same period, body weight increased nearly 70 per cent. During the following three months food intake and body weight increased slowly and at an almost constant rate. Running activity showed 4-day estrous peaks and otherwise only minor fluctuations.

In almost every case, the drugs and hormones under examination had no significant demonstrable effect during the period of administration. In marked contrast, immediately after the cessation of drug administration, some of the rats developed abnormal patterns in spontaneous running activity, food intake, and the functioning of the reproductive system. Other treated rats did not show such changes until several months later. In either case the abnormalities were manifested as sharply separated periods of activity and relative inactivity, usually accompanied by marked variations of food intake. Vaginal smears revealed simultaneous variations in functioning of the reproductive system.

Figure 2 shows the record of one of our experimental rats before, during, and after a 116-day feeding period on a low concentration of sulfamerazine. The graph presents daily running activity, food intake and body weight in 5-day averages (thus minimizing the effects of the estrous cycles); it also shows daily readings of cornified cells in the vaginal smears. The period of sulfa feeding is indicated at the bottom of the chart by solid shading. During the 130-day pretreatment period the animal's food intake and body weight remained quite constant while spontaneous activity showed small fluctuations. During the 116-day feeding period with sulfamerazine, food intake showed only a slight decrease; the rat continued to gain weight; and its activity remained constant until during the last 30 days, when it dropped to a

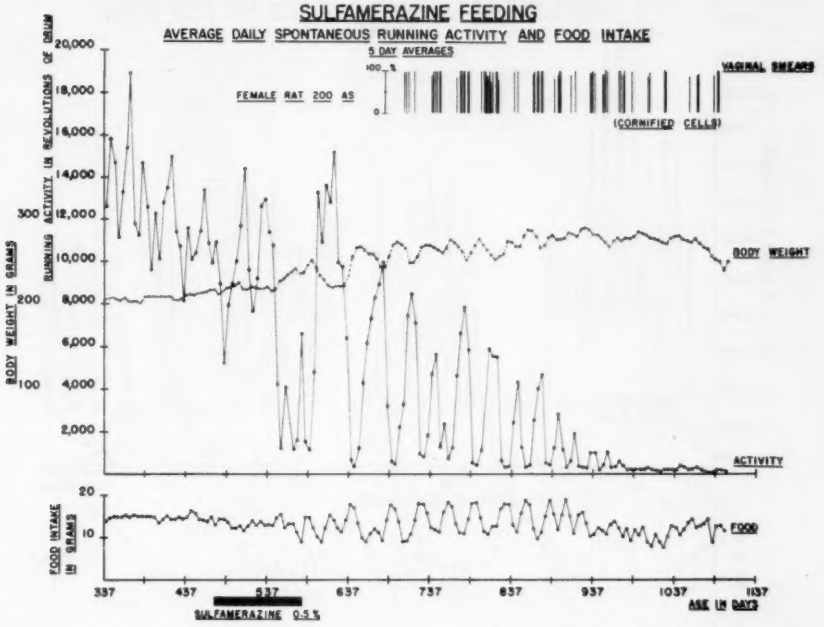

FIG. 2.—Graph showing daily spontaneous running activity and food intake in 5-day averages; percentage of cornified cells in vaginal smears; and weekly readings of body weight—before, during, and after treatment with sulfamerazine.

lower level. Throughout the treatment period the rat appeared entirely normal and unaffected by the ingestion of the sulfa drug.

However, the examination of the record from the discontinuation of the experimental diet to the animal's death over 500 days later reveals unmistakable evidence of permanent change. Average daily food intake almost at once began showing large regular excursions, both above and below the level maintained during the control and experimental periods; average daily activity likewise showed repeated swings, falling almost to zero, and then increasing to 6,000–8,000 revolutions; body weight similarly showed regularly recurring fluctuations. The first abnormal cycle had a duration of 58 days. The duration of the succeeding cycles progressively

became shorter, finally leveling off near 18 days; the height of the activity peaks and the amplitude of the excursions of food intake progressively became smaller until during the last four months of its life the rat became almost totally inactive and ate nearly constant amounts of food.

Figure 3 which gives part of the daily record of the same animal, starting almost immediately after the end of the sulfa feeding period, shows the cycles in more detail. It can be seen that each abnormal cycle consists of an active and an inactive phase, separated by sharp transitions. Since daily readings rather than 5-day averages

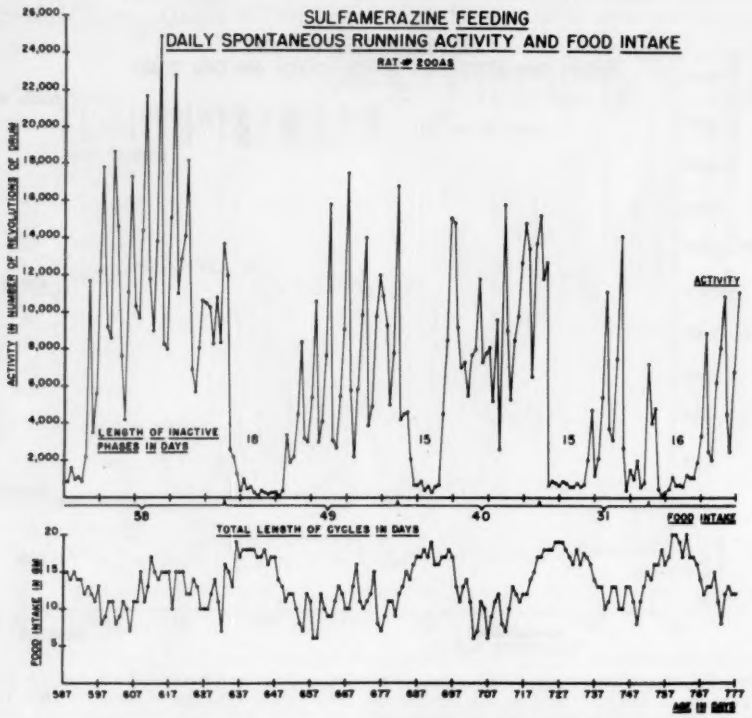

FIG. 3.—Graph for same sulfamerazine-treated rat showing daily spontaneous running activity and food intake. Record shows total length of cycle and length of inactive phase.

are shown, the estrous cycles in the active phases are clearly evident. While the active phases became ever shorter with time (decreasing from 40 to 2 days)the length of the inactive phases remained nearly constant (15–19 days)—as measured from the last peak of an active phase to the first peak of the following active phase. These abnormal activity cycles were simultaneously accompanied by inverse variations in food intake, showing remarkably even swings above and below the pretreatment level and with the maximum food intake consistently coinciding with the inactive phase of the activity cycle. The continuation of the record in Figure 4

shows that the cycles persisted almost throughout the remainder of the animal's lifetime. The rat died at an age of 1,105 days.

Table 1 shows that 5 of 25 rats, or 20 per cent, developed such abnormal and regular cycles after the termination of the sulfamerazine feeding.

Similar records were obtained by feeding various antithyroid drugs—thiourea, thiouracil, and propylthiouracil. In single doses these compounds are almost nontoxic to the rat, as they are to man; they may even be given in quite large doses without endangering life. That, however, they may do serious and permanent damage after having been administered in small doses for several months, was shown by the appearance of abnormal cycles in activity and food intake which developed only after the rats were returned to the regular stock diet. These cycles closely resembled those which followed sulfamerazine feeding.

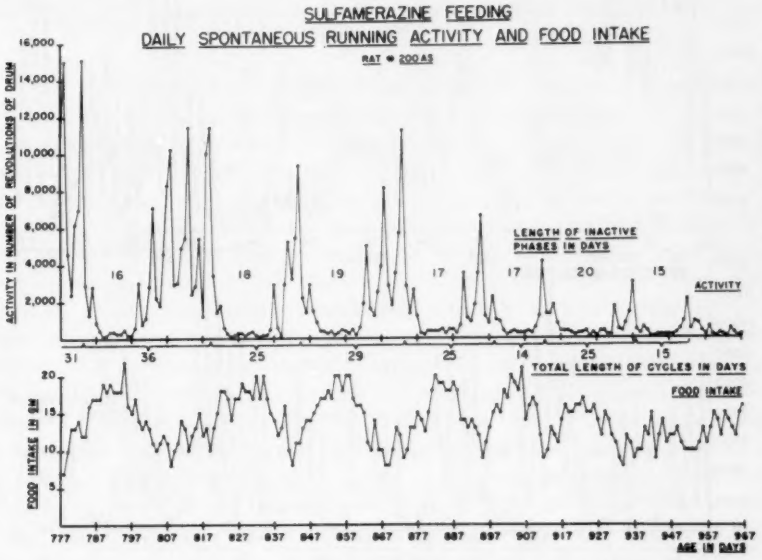

FIG. 4.—Continuation of record shown in Fig. 3.

Table 1 shows that after the end of the treatment with thiourea, 1 of 3, or 33.3 per cent, of the rats showed regular abnormal cycles; 4 of 5, or 80 per cent, of the thiouracil-fed rats, and 3 of 7, or 42.8 per cent of propylthiouracil-fed rats subsequently developed similar cycles. The abnormal cycles that followed treatment with sulfamerazine or the thioureas[1] closely resembled those that followed partial thyroidectomy or 1, 2 or 3 treatments with radioiodine I^{131}.[4]

Similar permanent abnormal cycles were also induced by prolonged feeding of the well-known and once widely used analgesic and antipyretic aminopyrine (Pyramidon), as may be seen in Figure 5. In single doses this drug also is practically nontoxic to the rat. It was fed in varying concentrations for 116 days (1 per cent for 18 days, 2 per cent 11 days, 1 per cent for 42 days, and 1.5 per cent for 45 days). During this time the rat became less active but showed no other untoward effects.

That, however, permanent change had been brought about by the prolonged feeding was shown by the appearance of abnormal activity cycles after the end of the treatment (at age 170 days). This rat has now been observed more than two years since the discontinuation of the aminopyrine feeding and its abnormal cycles are progressively becoming more regular with time. The total duration of the cycles has decreased from 54 to 18 days with a decrease in the length of the active phases (from 42 to 4 days) while the lengths of the inactive phases have tended to remain the same (9 to 13 days—peak to peak).

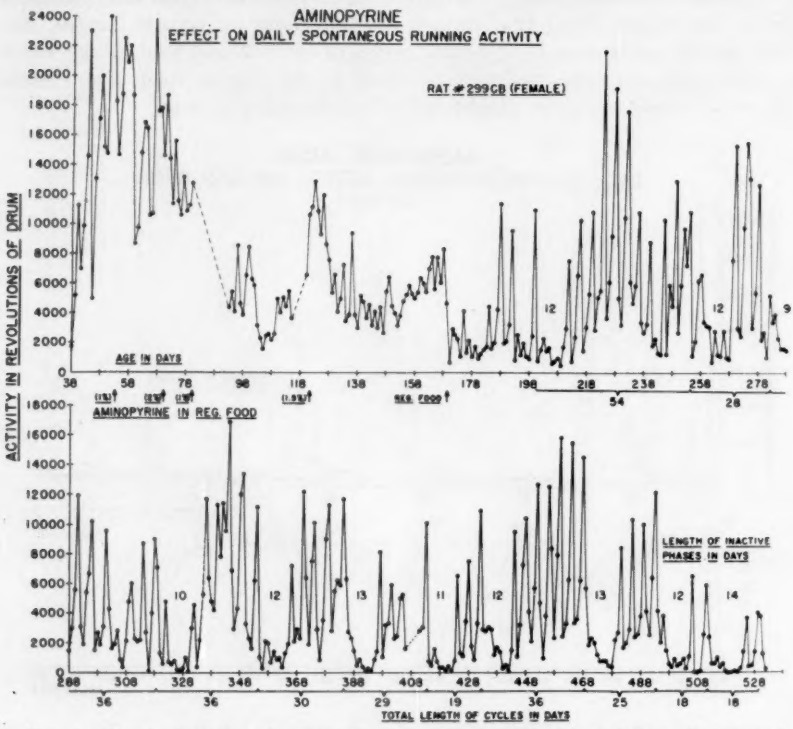
AMINOPYRINE
EFFECT ON DAILY SPONTANEOUS RUNNING ACTIVITY
RAT #299GB (FEMALE)
ACTIVITY IN REVOLUTIONS OF DRUM
AGE IN DAYS
AMINOPYRINE IN REG. FOOD
REG. FOOD
LENGTH OF INACTIVE PHASES IN DAYS
TOTAL LENGTH OF CYCLES IN DAYS

FIG. 5.—Graph showing daily spontaneous running activity of rat before, during, and after treatment with aminopyrine. Record shows total length of cycles and length of inactive phase.

Table 1 shows that 1 of 6, or 16.6 per cent, of the rats developed regular abnormal cycles after the end of the aminopyrine treatment. The cycles of this rat were quite different from those seen in the sulfa or thiourea-treated rats.

The end of a prolonged period of feeding with the sedative sodium barbital was followed by the appearance of marked fluctuations only in food intake; however, these showed only suggestive periodicity. Figure 6 gives the food intake record of one of these rats. This rat received sodium barbital in its diet in the following concentrations—0.1, 0.2, 0.3, and 1.0 per cent for 12 days, 28 days, 63 days, and 21 days, respectively). During treatment food intake remained fairly constant but soon after the end of treatment, marked fluctuations appeared.

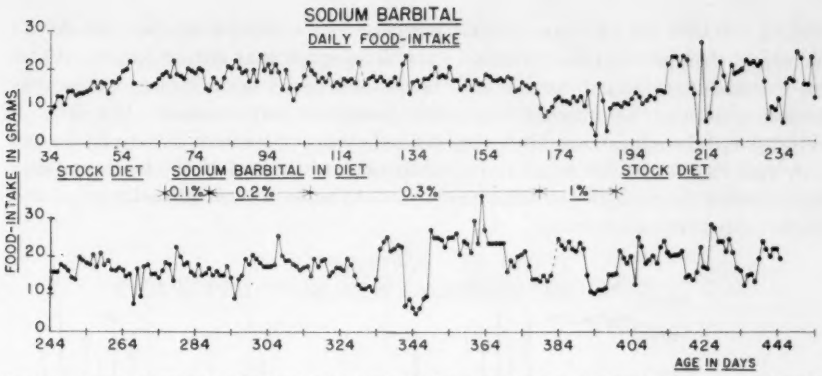

FIG. 6.—Graph showing daily food intake record for a rat before, during, and after treatment with sodium barbital.

Table 1 shows that 1 of 4 or 25 per cent of the rats manifested these changes of food intake after the termination of treatment on sodium barbital.

Prolonged treatment with cortisone acetate was followed in one rat by the appearance of very regular abnormal cycles in activity and food intake. Figure 7 shows this animal's daily spontaneous running activity, food intake, and body weight for 27 days before treatment, 105 days during treatment (cortisone mixture in diet 0.01 per cent for 63 days, followed by 0.04 per cent for 42 days) and for 239 days after termination of treatment. During the first 40 days on the 0.01 per cent concentration, the spontaneous activity remained on a high level, then for the last 20 days dropped to a lower level; food intake remained essentially the same; body weight continued to increase at a constant rate throughout the 63 days on this concentration. On the 0.04 per cent concentration, activity dropped almost at once to a low level where it remained for the rest of the 40 days; food intake decreased to a slightly lower level; body weight leveled off but did not decrease. Immediately after the cessation of the treatment, activity and food intake increased to their original high levels; body weight increased at a slow but constant rate. For the next 70 days, activity and food intake, water intake, body weight, and estrous cycles were all normal so that the rat appeared to have made a full recovery. Then quite suddenly, activity dropped almost to zero, food intake and body weight increased, and the estrous cycles disappeared. This was the beginning of an inactive phase of 20 days' duration. Then followed a series of inactive phases of almost exactly the same length but separated by progressively shorter active phases. Food intake increased during the inactive phases and dropped sharply a few days before the onset of the following active phase.

Table 1 shows that 1 of 3 rats developed regular abnormal cycles after the end of the cortisone treatment. The cycles of this rat differed from those seen in the aminopyrine and the sulfa or thiourea-treated rats. (These observations were made while studying with Dr. T. E. Van Metre, Jr., the effects of cortisone on growth.)

Even more regular abnormal cycles of spontaneous running activity were shown by rats some months after the administration of the female sex hormones, estradiol and progesterone. Figure 8 shows a record of the activity cycles produced by

feeding estradiol for 141 days (0.0001 per cent for 74 days, 0.001 per cent for 34 days, and 0.01 per cent for 33 days). It will be noted that during treatment this rat was about one-fourth as active as before and it did not show regular estrous cycles; otherwise, to ordinary inspection, it was entirely normal. The drop in activity and the appearance of sharply defined cycles with bursts of activity lasting only one day, every 18 to 22 days, subsequent to the end of the treatment and lasting until the animal's sacrifice many months later, indicates fundamental and presumably permanent change.

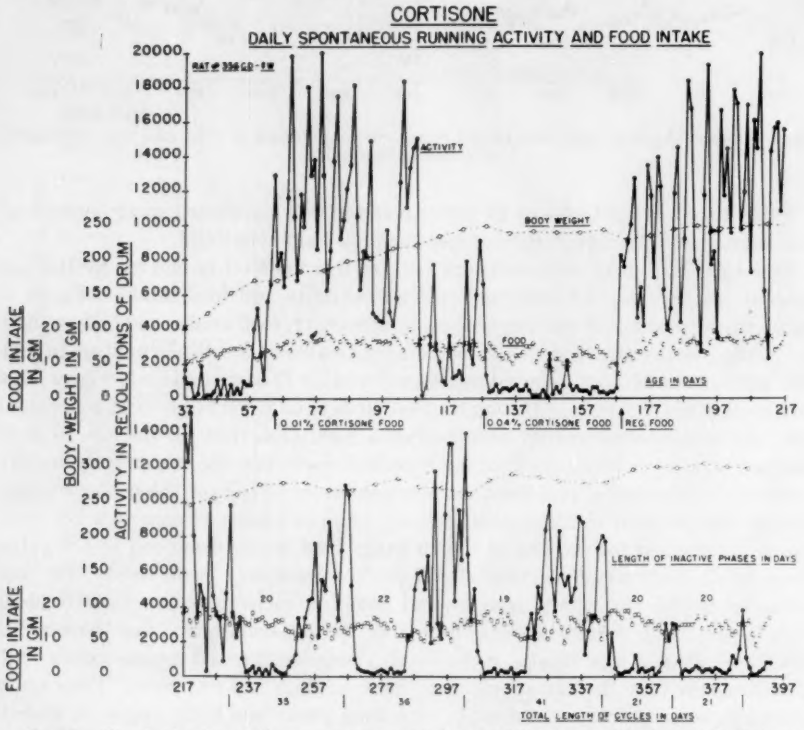

FIG. 7.—Graph showing daily spontaneous activity, food intake, and body weight before, during, and after treatment with cortisone acetate. The record gives the total lengths of the cycles and the lengths of the inactive phases in days.

Table 1 indicates that 2 of 3 rats or 66.6 per cent show regular abnormal cycles after the end of the estradiol treatment. These abnormal cycles differ from any of those seen in the rats treated with sulfa or thioureas, aminopyrine or cortisone. The lengths of the inactive phases, however, were much the same as in the sulfamerazine and in the cortisone-treated rats.

Prolonged feeding with progesterone resulted in the appearance of activity cycles during, as well as after treatment in the one rat on which observations were made. Figure 9 shows the record of this rat that received anhydrohydroxyprogesterone for a total of 492 days and was observed 239 days thereafter. Treatment was started at the age of 110 days and concentrations were changed according

to the following schedule: 0.025 per cent for 33 days, 0.05 per cent for 63 days, 0.15 per cent for 258 days, 0.3 per cent for 9 days, stock diet for 11 days, 0.3 for 128 days. Abnormal cycles showing both active and inactive phases appeared almost at once after the start of the treatment and were maintained throughout the hormone administration. During this period the length of the active phases decreased at a steady rate from 54 days to 1, while that of the inactive phases ranged from 15 to 28 days. During the 238 days of the post-treatment period the length of the in-

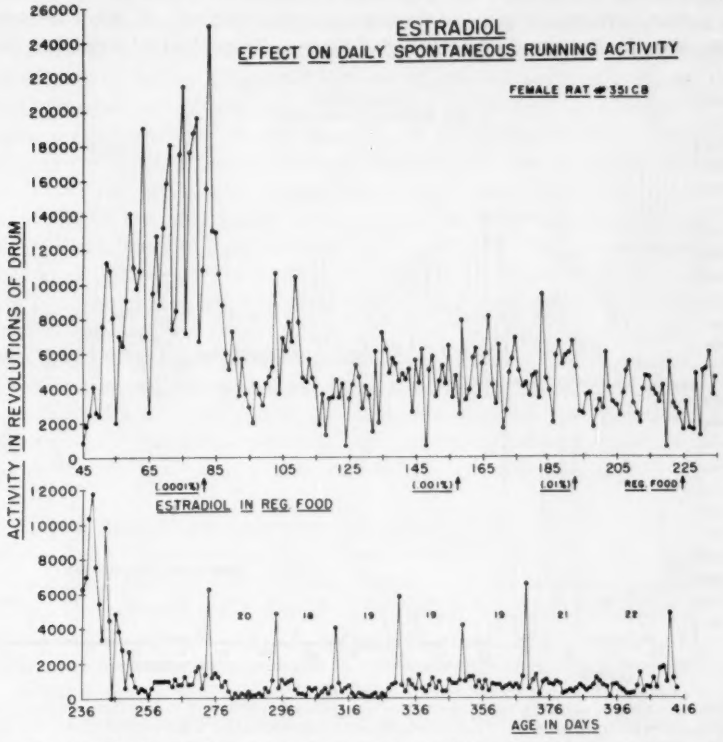

FIG. 8.—Graph showing spontaneous running activity of rat before, during, and after treatment with estradiol. The record gives the length of the cycles in days.

active phases increased slowly to 33 days. At autopsy, a large tumor of the pituitary was found. This tumor itself many have been responsible for this cycle since previously in an untreated rat a pituitary tumor was found to produce very regular cycles—however, the cycles were shorter than those of the progesterone-treated rat.[1]

These observations have demonstrated that prolonged administration of several commonly used drugs and hormones may produce lasting changes in the organism, as manifested by abnormal cycles in spontaneous running activity, food intake, functioning of the female reproductive system, and in some instances in water intake. Had daily readings been made of liver function, body temperature, and pro-

portion of the various types of leucocytes, it is possible that equally regular and permanent changes may also have been found in these functions.

At the present time we are not able to produce abnormal cycles with any one of the drugs or hormones at will. Rats treated with thiouracil showed the highest proportion of abnormal cycles—80 per cent—but the numbers are too small to be significant. The over-all proportion of rats showing abnormal cycles is 33.3 per cent. In some instances abnormal cycles have followed treatment with minimal doses whereas cycles were not produced by treatment with higher doses. This was particularly true of some of the rats on sulfamerazine. In other instances the cycles were produced only by the higher doses. From these observations on small

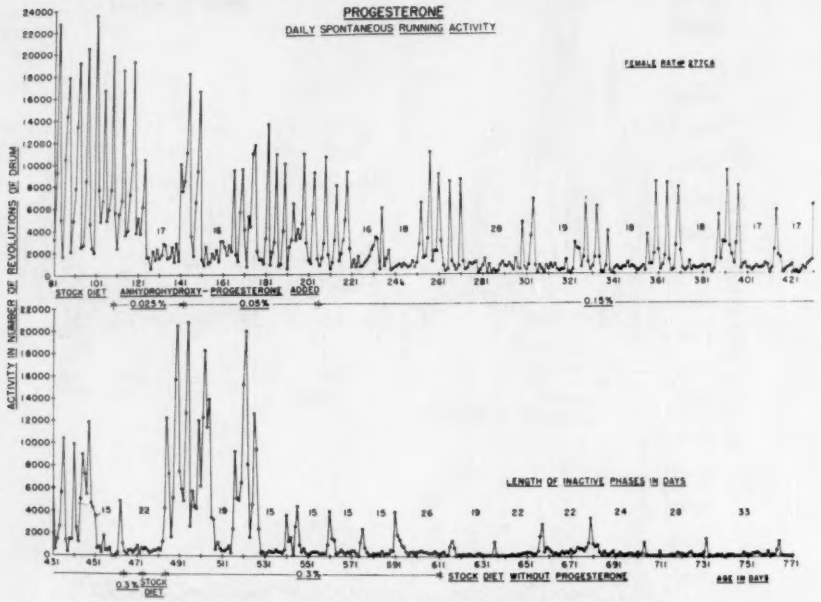

FIG. 9.—Graph showing daily spontaneous running activity of rat before, during, and after treatment with progesterone (anhydrohydroxy-progesterone). The record gives the length of the inactive phases of the cycles in days.

numbers of animals it was not possible to draw any conclusions about the significance of length of treatment. In a few instances regular abnormal cycles followed only 40–50 days of treatment. In any case, there can be no doubt about the pathological nature of these cycles since, as mentioned above, no similar cycles were found in the many thousands of rats that have been observed in this laboratory under identical conditions during the past 35 years.

The cycles described here differ essentially from those due to infection with the plasmodium in malaria or the spirochete in relapsing fever, which depend on periodic evolution of the infecting agents. The cycles herein described differ also from those resulting from the introduction of a foreign protein into the body, such for instance as horse serum. Najjar has recently shown that the introduction of foreign protein

results in a series of successive steps in which the antigen-antibody combination acts as a new antigen and stimulates a new antibody reaction.[5] In this way the foreign protein is gradually degraded; presumably the cycles are expressions of successive reactions of the body to the new antigen-antibody combination. Cycles of this type have been noted particularly in scarlet fever.[6] The cycles induced by parasitic infestation disappear when the parasite is destroyed or eliminated. Similarly the cycles induced by the introduction of foreign protein disappear with the neutralization of the antigen. In both cases the cyclic phenomena are transient and dependent on the presence of the exciting agent; in neither case do the cycles depend on permanent alteration of the tissue cells.

In contrast, the cycles being reported here may depend on permanent changes in cells either in the nervous system or in the endocrine glands or in the blood forming organs, such as bone marrow—the last possibility was suggested by the fact that we know of many instances of permanent agranulocytosis in man that have followed prolonged treatment with sulfa drugs, thiouracils, aminopyrine, or barbital.[7] The affected tissues are not actually destroyed; rather their function is impaired, resulting in a disturbance of their intricate interregulation. As a consequence, an alternating series of under-actions and over-actions seem to occur which manifest themselves in cyclic alterations. In other words, the homeostatic mechanisms no longer operate smoothly.

Attention may be drawn at this place to the fact we have previously reported the production of abnormal cycles with other characteristics and frequencies by surgical interference with the pituitary gland, especially the stalk or posterior lobe,[9] by partial inactivation of the thyroid gland through surgery or radioiodine treatment,[1, 4] by lesions of the brain stem or cortex, and finally by subjecting rats to severe stress.[8]

On the basis of these observations on rats the possibility must be considered that the cessation of administration of a drug or hormone after prolonged treatment may also produce lasting effects in man; further, that the existence of such changes may not be detected without the aid of special measurements made over long periods of time.

That abnormal cycles in behavior and metabolism similar to those seen in our rats may appear in man is well known from daily observations made in medical and psychiatric clinics over long periods of time of patients with periodic physical and mental illnesses. Thus, in the literature there are records of many patients in whom very regular cyclic changes were demonstrated, primarily in physical functions, such as body temperature, blood cell counts, fluid content of tissues, etc. Reimann reported a number of such patients[10] and more recently, two monographs on periodic illness have been published in France by Mamou[11] and Sebaoun.[12] Many articles on this subject have also appeared in recent years in the medical journals of almost all countries of the world, especially in those bordering the Mediterranean.

We have collected the records of over 400 patients with one form or other of periodic illness manifesting itself primarily through physical symptoms. We also have records of almost as many patients in whom equally regular cyclic changes were primarily evident in mental and emotional functions, such as concentration, mood, etc. Some of the most regular and impressive of these cycles were reported by

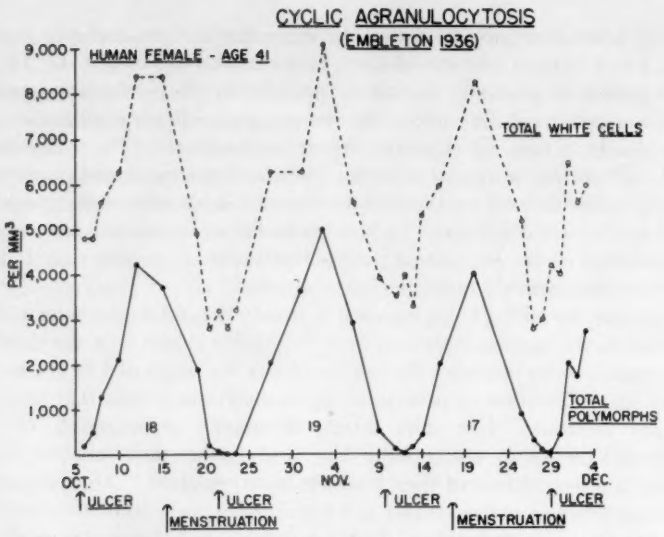

FIG. 10.—Record of total leucocytes and polymorphs of 41-year-old female. Record gives lengths of cycles in days and indicates the occurrence of mouth and anal ulcers and of menstruation.

Gjessing.[13, 14] If daily records could be taken on an entire population, the existence of unsuspected abnormal cycles might be revealed in many more individuals.

To illustrate the nature of these cycles a few examples will be presented. Figure 10 gives the record of the number of total white cells and polymorphs in a patient with cyclic agranulocytosis.[15] It also shows the menstrual cycle and the periodic appearance of ulcers in the mouth. The total polymorphs fluctuated between zero and over 4,000 per m^3 in regular cycles with a frequency of 17–19 days. These cycles had no relation to the menstrual periods. Oral and anal ulcers regularly developed shortly after each sharp reduction in the number of polymorphs. We have several similar records.

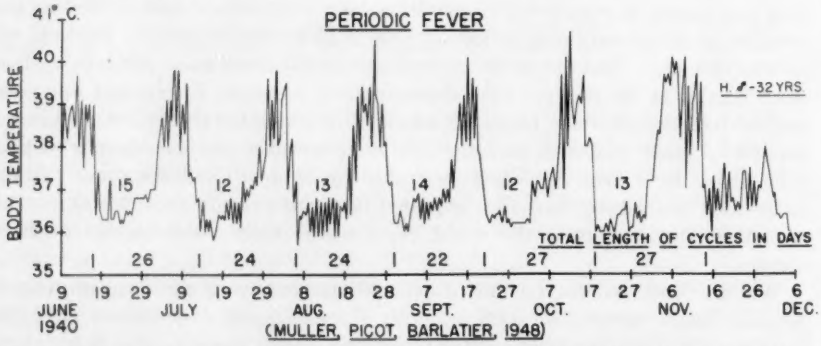

FIG. 11.—Daily morning-evening temperatures of 32-year-old male. Record shows lengths of total cycles and lengths of normal intervals between periods of fever.

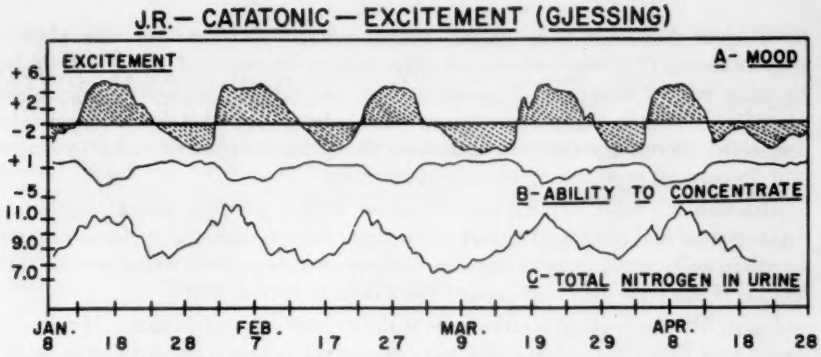

FIG. 12.—Behavior and metabolism record of catatonic-schizophrenic patient (Gjessing) showing estimated excitement and ability to concentrate, as well as total nitrogen in urine. The cycles were 20 days in length.

Such cycles many show up also in body temperature, as is seen in Figure 11.[16] In this patient the temperature ranged from about 36° to 40°C in regular cycles with a frequency of 22–27 days. Similar cycles of physical illness may appear in the function of almost every organ of the body. The known frequency ranges from 2 days to 60–80 days. Nearly all, if not all, of these patients manifest at least some mental or emotional changes.

Gjessing's studies of catatonic-schizophrenic patients, with detailed daily records over long periods of time, have revealed the fact that equally regular cycles occur in mental and emotional functions of some psychiatric patients.[14] Figure 12 gives the record of one of his patients with catatonic excitement. The top graph represents an estimate of mood, the second the ability to concentrate, while the third graph gives daily record of total urinary nitrogen. In this case the cycles had an average duration of 20 days.

Figure 13 shows the daily record of another one of Gjessing's patients—a catatonic-schizophrenic with cyclic stupors.[12] The top graph gives the estimate of the presence and depth of the stupors; the second shows spontaneous activity in bed, as measured by an actograph (this patient remained in bed throughout); while the

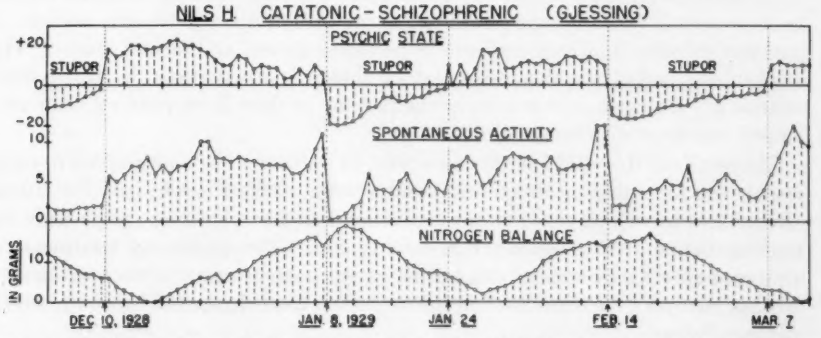

FIG. 13.—Behavior and metabolism record of catatonic-schizophrenic with periodically recurring attacks of stupor. The cycles averaged 40 days in length.

third again shows the daily record of total urinary nitrogen. In this patient, stupors lasting 17–21 days recurred with an average frequency of 40 days, separated by fairly normal intervals. It is noteworthy that this patient and the one whose record was shown in Figure 13 both responded to treatment with thyroxine and later desiccated thyroid powder. In both cases the cycles disappeared under treatment and the patients were able to return to simple jobs.

Although the most striking manifestations in this group of patients appear as cyclic mental and emotional disturbances, there are simultaneous cycles in physical function, as for instance in nitrogen excretion as well as in body temperature. The lengths of the reported cycles range from 2 days to several years.

Again, little or nothing is known about the underlying mechanisms. It certainly seems very likely that some cycles have one origin, others quite a different origin, as is true in our experimental rats.

At present we do not know whether any of the reported abnormal cycles in behavior, mood, and metabolism in human beings followed upon prolonged treatment with any drugs or hormones. So far as we know, this possibility has not hitherto been considered. We do know that some patients treated with sulfa and antithyroid drugs have become psychotic during treatment,[17] and in some instances the symptoms only appeared a few days or weeks after the end of the treatment;[17–19] it is also known that some of the drugs that produced cycles in spontaneous activity,[1] etc., in our experimental rats also produced agranulocytosis in some patients through a depressive effect on bone marrow.

Summary.—It has been demonstrated in rats that lasting effects in the form of abnormal cycles of spontaneous running activity, food and water intake, and functioning of the reproductive tract may follow upon the discontinuance of drug or hormone (sulfamerazine, thiourea, thiouracil, propylthiouracil, aminopyrine, sodium barbital, cortisone acetate, estradiol, or progesterone) after its prolonged administration. In some instances the abnormalities are manifest at once after the termination of treatment; in others there may be a delay of many months before any pathology is demonstrable. The changes involve both behavior and metabolism; in nearly every case they persist throughout the remaining life of the animal.

Instances of regular abnormal cycles of behavior, mood, and metabolism, similar to those seen in rats after the end of treatment with drugs or hormones, are seen in patients suffering from various forms of periodic physical and mental illness. The mechanisms underlying the production of these abnormal cycles in man remain unknown. Likewise, it is unknown whether any of them have resulted from prolonged treatment with drugs or hormones.

The results of this study on rats, however, do warrant a recommendation to those responsible for screening drugs and hormones for medical use to look for lasting after-effects as well as concomitant side-effects; and to physicians responsible for treating patients to consider the possibility that although during treatment no untoward effects may appear, damage may appear after the end of the treatment—damage that may manifest itself in subtle pathological changes in behavior, mood, and metabolism.

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† On leave at the Institute for Advanced Study, Princeton, New Jersey, 1957.

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*REPRODUCTIVE CYCLES IN AN EQUATORIAL SPARROW**

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Read before the Academy, April 29, 1959

The male avian reproductive system undergoes cycles of activity in which there are periods of reduced, nonfunctional gonads alternating with those when the active testes are 150 times the size of the resting stage. Such cycles occur in essentially all wild species that have been investigated. Control of the cycle by the gonadotropic hormones of the pituitary has been demonstrated, a control that parallels the especially well-documented situation in mammals of pituitary regulation of reproductive periodicity.

In every instance in birds in which manipulation of the cycle has been attempted by experimental light treatment, it has been found that greater day length stimulates the pituitary-gonad mechanism. On the other hand there are periods following maximum activity, and also periods in the maturing of individuals, when the mechanism is refractory to the normally stimulating effects of light increments. These seemingly necessary periods of rest and organization have a strong tendency to alternate with recrudescent phases and constitute the basis for ascribing an innate rhythmic attribute to the mechanism. The general conclusion has been drawn that the annual reproductive cycles of species of temperate and high latitudes is the result of this general rhythmic tendency coerced by seasonal photoperiodism so that the onset of the breeding season is precisely timed in adaptation to the average annual development of favorable ecologic conditions for the species; the breeding effort that ensues thus can achieve maximum success.

As one examines species living at progressively lower latitudes, where seasonal photoperiodism is less extreme, many instances of the influence of factors other than day length become apparent. Thus even at latitude 38° some species have breeding seasons during the shortest days of the year in adjustment to rainfall patterns and the related plant and insect food sources.[1] In a few instances at least the actual or proximate stimuli to recrudescence have been traced to factors such as flowering of plants or availability of plant growth[2] or to the onset of rains. These stimuli other than light are best revealed in desert areas where they are variable in timing from year to year and where recrudescence therefore cannot possibly be keyed to any phase of photoperiodism.

Nevertheless the prevailing influence of photoperiods is detected, through critical correlations, in an equatorial direction as far as latitude 10°N where most species breed during the longer days of the year following the vernal equinox.[3] This is true both in wet and dry belts and among species that are partly influenced by seasonal moisture conditions.

In equatorial latitudes, between 5°N and 5°S, the photoperiod fluctuations are so reduced as to have no physiologic effect on reproductive rhythm. One may expect, then, (1) that innate cyclic tendencies will be expressed in some species without the coercion of photoperiodism, (2) that such species may as species show continuous breeding because the normal variability in length of the presumptive individual cycles would put these individuals out of phase with respect to one another, and (3) that other species that inhabit areas with strong rainfall cycles will be coerced into exhibiting coordinate, restricted breeding seasons by evolving sensitivities to some one of a variety of proximate stimuli arising directly or indirectly from rainfall.

As a result of previous work in Colombia, South America, at equatorial latitudes in an area with weak rainfall cycles, it was found that in eight of ten sample species breeding condition in males could be encountered in all segments of the year.[4] But we discovered also, as evidence from equatorial Africa had already partly shown,[5] that adult males of these species undergo testis regression to a point fully equivalent histologically to the state of winter dormancy of north-temperate birds. It was thus evident that individuals underwent some type of cycle even in the continuously breeding species.

The purpose of the investigation of 1958 in Colombia was to ascertain in such a continuously breeding bird the number of cycles and the duration of each phase of

the cycle in a given individual through a twelve-month period. The species selected for study was *Zonotrichia capensis*, a congeneric relative of the North American Sparrows of the genus *Zonotrichia* on which much of the experimental work on photoperiodism and breeding physiology has been conducted.[6, 7, 8] To achieve significant results on *Zonotrichia capensis*, free-living individuals marked for identification needed to be followed by means of observation and by periodic recapture to ascertain the occurrence of stages of testis enlargement and regression.

Methods and Procedure.—A research station was established in an area of dense population of *Zonotrichia capensis* on the crest of the Western Andes, 6,500 feet elevation, west of the city of Cali, Colombia, at latitude 3°30′N. This research center was maintained from February 8, 1958, to January 23, 1959. In the course of the year 160 individuals were captured and individually color marked on an area of four acres. The most critical results were derived, however, from the subsequent history of the first 50 birds captured in the initial three weeks. These latter consisted largely of resident adult and postjuvenal individuals, in which there was good survival, of about 78 per cent, through the ensuing ten or eleven months.

Breeding level in males is regarded as a stage of testis development in which mature sperm are present either in bundles or free in the lumen of the seminiferous tubules. If sperm are not free, it may safely be assumed that they can be so freed within five to ten days under the stimulus of courtship and nesting behavior of the pair and that the male is therefore in potential breeding condition. In the regressed testis spermatogonia and primary spermatocytes are present but only a few of the latter show synapsis of the chromosomes. By correlation studies of the linear dimension of the testes and microscopic sections carried out on birds collected in areas adjacent to that occupied by the living marked population, it was determined that testes 6 to 9 mm in length are in potential breeding condition and that testes 1.5 to 2.5 mm in length were in inactive state. Testes 2.5 to 5.0 mm in length if they are firm or turgid are in various stages of the maturation divisions which implies a prior period of inactivity. The decline from active state is very rapid and the testis in this transition is soft and usually distinctively yellow.

Further correlation studies among the 80 birds autopsied showed that a cloacal gland development represented by external diameters of 5 to 8 mm of the cloacal protuberance invariably reflected a testis in potential breeding condition. Moreover, birds that were engaging in steady territorial defense song always possessed an active testis. The converse situations, however, did not hold. Reduced cloacal glands and absence of song may at times accompany a fully enlarged testis.

A critical procedure was the recapture of individuals and the performing of a laparotomy whereby the internally situated testis could be measured with calipers. Following closure of the abdominal wall and recovery from anesthesia the bird, within an hour, could be returned to the field, where it would at once resume its singing and nesting behavior if it were in an active state. This operation was performed as many as three or four times on some individuals without any ill effects and it was particularly important in establishing periods when the testes were in regressed or early recrudescent stages. The uncertainties of survival in the wild and of recapture at the proper periods for laparotomies were factors on which we gambled but with which we had good fortune.

Age determination of individuals was made by observing presence and progressive disappearance of the ventrally streaked juvenal plumage. All identifiable remnants of such plumage are lost by 80 days of age. Up to eight months of age, and occasionally to ten months, evidence of immaturity persists in the skull in the form of progressively reduced areas of translucent bone in the frontal region; these "windows" are later closed through full separation of the two layers of this bone by an air space and supporting trabeculae. This skull area may be repeatedly examined by operating without anesthesia to slit the thin overlying skin and expose the bone; suturing with scotch tape permits immediate return of the bird to the field. Age in the period from three to nine months can thus be gauged approximately by measuring the size of the frontal "window."

By correlation of all evidence derived from operations, external features of the plumage and the cloaca, and behavior involving song, copulation, and actual participation in nesting, the reproductive state could be ascertained in a number of males over a ten to twelve months' period.

Results.—The evidence indicates a full reproductive cycle, involving one high and one low point, of five to seven months' duration, the normal length being six months. Case histories of 29 postjuvenal and adult males that reflect status over a period of six months or more in no instance offer the slightest suggestive evidence of appreciably longer cycles. Moreover, the like records of 27 females in no instance show sustained breeding activity for more than six months without rest periods.

The positive record of cycles in males consists of 17 timed cycles attested by laparotomies for the low points and by laparotomies and/or positive cloacal or behavioral evidence for the alternating high points. There are five additional cycle records for which the evidence for the low points is indirect, consisting of conspicuous cessation of song and/or heavy involvement with molt.

The most decisive records are those derived from five males in which two complete cycles were traced, thus clearly demonstrating the double cyclic situation for the annual period. The records of these are summarized in Table 1. Herein the

TABLE 1

RECORDS OF CYCLES OF FIVE WILD MALES OF *Zonotrichia capensis*

Individual	High	1st Low Point	High	2nd Low Point	High
466	C	Mar. 2	L, C, N	Aug. 25	C, N
472	...	Mar. 20 (Apr. 7)	L, C, N	Aug. 26	C, N
480	L, C	Mar. 16	L, C, N	Oct. 20	C
484	L, C	Mar. 20 (Apr. 8)	C, N	Nov. 3	...
806	...	Mar. 8	C	Sept. 8	C

critical low points are determined by laparotomies. If the operation revealed a testis in middle stages of recrudescence, the low point was extrapolated from this and the actual date of the operation placed in parentheses. The fully resting testis state is apparently maintained for only two or three weeks at most and thus serves as a convenient reference point. The alternating periods of full breeding potential are attested in these birds by laparotomies (L), and/or greatly enlarged cloacal protuberances (C), and participation in nesting (N).

The length of the sustained breeding condition or plateau was determined rather closely in ten adult birds. These are best expressed as minimum durations

based on positive evidence. They range from three to six months, but eight of the ten are in the range of 3 1/2 to 4 1/2 months. Maximal possible periods might be estimated by extrapolation from intervening lows but would in no instance be more than two or three weeks greater than the proved minima.

In immatures undergoing their first testicular enlargement, the plateau may be shorter. In one instance it was proved to be only 2 2/3 months and similar short durations were suggested purely by behavioral evidence in several other immature individuals. Such short plateaus were never found in adults.

The interval of rest could be measured only as the gap between the end of one high plateau and the beginning of the next, owing to the rapidity of regression and the briefness of actual rest (less than a month) and recrudescence. To ascertain and time the progress of these rapid changes would have necessitated more frequent captures and operations than the circumstances and the recovery of the birds would permit. The maximum time for the regression and recovery interval ascertained from six histories ranged from six weeks to three months, the latter probably longer than real; the normal period was two months.

The age in months at which young males first attain breeding condition was found to be as follows in seven individuals with adequate records: 5, 6, 7, 7 1/3, 8, 9 1/3, 11 1/3. There is a very small chance that in the last two instances a very brief recrudescence and subsidence could have occurred short of nine to eleven months and been undetected but it could not have been of such duration as actually to have permitted nesting. Although we were not fortunate enough to find any of these particular males actually engaged in nesting, two females at calculated ages of 4 1/2 and 9 1/2 months had laid clutches of eggs. In the first instance if we allow for some delay in initiating or completing the postjuvenal molt beyond the normal chronology, the age of breeding might have been five months, but not more.

To be detailed in another paper are full data on weather and on the statistics which show when the largest numbers of individuals are at breeding level. Briefly it may be indicated here that the modal occurrences of breeding fall in May, June, and July and in December, January, and February. These months represent the ends of the wetter seasons and the early parts of the ensuing drier periods; the heaviest rains are in April and May and in late October, November, and December (Table 2). Thus the peaks of breeding are related to and in some

TABLE 2

WEATHER RECORD AT 4 KM NW SAN ANTONIO, 6,500 FT, VALLE, COLOMBIA
Monthly averages of maximum and minimum temperature (°F) and total rainfall, February 8, 1958 to January 23, 1959

	Minimum	Maximum	Rainfall, in.
January	57.9	70.0	1.1 (23 days only)
February	58.5	71.5	3.065(20 days only)
March	58.6	72.9	5.51
April	58.2	71.4	7.37
May	58.7	72.1	8.05
June	58.6	73.8	1.13
July	57.8	72.5	1.29
August	57.6	73.4	3.47
September	57.3	72.7	1.16
October	56.9	70.7	7.60
November	56.7	68.6	4.95
December	56.7	68.4	6.49
			51.23 (1 yr. less 15 days)

way induced by the rainfall cycle but not by the weak traces of photoperiodism, for it is to be observed that one peak spans the summer solstice (for 3 $^1/_2$°N latitude) and the other the winter solstice. It should be re-emphasized, however, that birds in breeding state and successful nesting occur in every month of the year.

Conclusion and Summary.—In equatorial species of birds that exhibit potential breeding state at all periods of the year, individual males undergo cycles with maxima and minima equivalent in degree of difference in physiological level to those of their temperate zone relatives. In one such species, *Zonotrichia capensis*, the first of its kind to be studied intensively, the cycles of the individual average six months in duration and two complete cycles are manifest each year. The high plateau of breeding potency in males normally lasts four months and the intervening regression, rest, and recrudescence of the testis occupies two months.

The six-month cycle in *Zonotrichia capensis* is an expression of innate cyclic tendency. It is uncoerced by small variations in photoperiod and is only incompletely controlled by the seasonal occurrence of rainfall, which however does appear to induce a majority of the population to engage in breeding in the late parts of each of the two wetter periods of the year.

Young males attain the breeding plateau at five to eleven months of age. Young females may actually engage in nesting when five months old.

The variable age of attainment of breeding potential and the individual variability of the innate cycle of the adult males is responsible for the production of some young in every month of the year.

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Williams, Roger John, 1946 (5), Clayton Foundation, Biochemical Institute, University of Texas, Austin 12, Texas

Willier, Benjamin Harrison, 1945 (8), Department of Biology, Johns Hopkins University, Baltimore 18, Maryland

Wilson, David Wright, 1955 (14), Department of Biochemistry, School of Medicine, University of Pennsylvania, Philadelphia 4, Pennsylvania

Wilson, Edgar Bright, Jr., 1947 (5), Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge 38, Massachusetts

Wilson, Edwin Bidwell, 1919 (3), 42 Brington Road, Brookline 46, Massachusetts

Wilson, Perry William, 1955 (7), Department of Bacteriology, University of Wisconsin, Madison 6, Wisconsin

Wilson, Ralph Elmer, 1950 (2), 605 Acacia Avenue, Corona del Mar, California

Wilson, Robert Erastus, 1947 (4), 135 South La Salle Street, Chicago 3, Illinois

Wilson, Robert Rathbun, 1957 (3), Laboratory of Nuclear Studies, Cornell University, Ithaca, New York

Winstein, Saul, 1955 (5), Department of Chemistry, University of California, Los Angeles 24, California

Wintersteiner, Oskar, 1950 (14), Squibb Institute for Medical Research, New Brunswick, New Jersey

Wolfrom, Melville Lawrence, 1950 (5), Department of Chemistry, The Ohio State University, Columbus 10, Ohio

Wood, Harland Goff, 1953 (14), Department of Biochemistry, School of Medicine, Western Reserve University, Cleveland 6, Ohio

Wood, William Barry, Jr., 1959 (10), School of Medicine, Johns Hopkins University, 725 North Wolfe Street, Baltimore 5, Maryland

Woodring, Wendell Phillips, 1946 (6), United States Geological Survey, Washington 25, D. C.

Woodward, Robert Burns, 1953 (5), Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge 38, Massachusetts

Woodworth, Robert Sessions, 1921 (12), 400 West 119th Street, New York 27, New York

Woolley, Dilworth Wayne, 1952 (14), Rockefeller Institute, New York 21, New York

Wright, Sewall Green, 1934 (8), Department of Genetics, University of Wisconsin, Madison 6, Wisconsin

Wu, Chien-Shiung, 1958 (3), Department of Physics, Columbia University, New York 27, New York

Wulf, Oliver Reynolds, 1949 (13), United States Weather Bureau, Gates and Crellin Laboratories of Chemistry, California Institute of Technology, Pasadena 4, California

Wyckoff, Ralph Walter Graystone, 1949 (5), Departments of Bacteriology and Physics, University of Arizona, Tucson 25, Arizona

Yoder, Hatten Schuyler, **Jr.,** 1958 (6), Geophysical Laboratory, Carnegie Institution of Washington, 2801 Upton Street, N. W., Washington 8, D. C.

Yost, Don Merlin Lee, 1944 (5), California Institute of Technology, Pasadena 4, California

Young, William Gould, 1951 (5), Department of Chemistry, University of California, Los Angeles 24, California

Zacharias, Jerrold Reinach, 1957 (3), Department of Physics, Massachusetts Institute of Technology, Cambridge 39, Massachusetts

Zachariasen, Frederik **William** Houlder, 1949 (3), Division of the Physical Sciences, University of Chicago, Chicago 37, Illinois

Zariski, Oscar, 1944 (1), Department of Mathematics, Harvard University, 2 Divinity Avenue, Cambridge 38, Massachusetts

Zener, Clarence Melvin, 1959 (4), Westinghouse Research Laboratories, Beulah Road, Churchill Borough, Pittsburgh 35, Pennsylvania

Zimm, Bruno Hasbrouck, 1958 (5), General Electric Research Laboratory, Schenectady, New York

Zinn, Walter **Henry,** 1956 (4), General Nuclear Engineering Corporation, P. O. Box 245, Dunedin, Florida

Zirkle, Raymond Elliott, 1959 (8), Committee on Biophysics, University of Chicago, 5640 Ellis Avenue, Chicago 37, Illinois

Zworykin, Vladimir **Kosma,** 1943 (4), RCA Laboratories, David Sarnoff Research Center, Princeton, New Jersey

Number of Members July 1, 1959: 599.

MEMBERS EMERITI

Albright, Fuller, 1952 (10), 271 Goddard Avenue, Brookline 46, Massachusetts

Anderson, John **August,** 1928 (2), P. O. Box 332, Pasadena 17, California

Bailey, Irving **Widmer,** 1929 (7), Harvard University Herbarium, 22 Divinity Avenue, Cambridge 38, Massachusetts

Coble, Arthur Byron, 1924 (1), Lykens Hotel, Lykens, Pennsylvania

Coolidge, William **David,** 1925 (3), 1480 Lenox Road, Schenectady 8, New York

Goodpasture, Ernest William, 1937 (10), Box 53, Williamsport, Tennessee

Mason, Max, 1923 (3), 1035 Harvard Avenue, Claremont, California

Saunders, Frederick Albert, 1925 (3), South Hadley, Massachusetts

Schultz, Adolph Hans, 1939 (11), Anthropologisches Institut, Sempersteig 3, Zurich, Switzerland

Stadie, William Christopher, 1945 (14), 817 Maloney Clinic Building, 36th and Spruce Streets, Philadelphia 4, Pennsylvania

Tyzzer, Ernest **Edward,** 1942 (10), 175 Water Street, Wakefield, Massachusetts

Vandiver, Harry **Shultz,** 1934 (1), Box 7881, University of Texas, Austin 12, Texas

Number of Members Emeriti July 1, 1959: 12.

FOREIGN ASSOCIATES

The number in parentheses following the year of election indicates association within the sections of the National Academy of Sciences.

Adrian of Cambridge, Edgar Douglas, Baron, 1941 (9), Trinity College, Cambridge, England

Alexandroff, Paul A., 1947 (1), Mathematical Institute of the Academy of Sciences of the U.S.S.R., Bolshaya Kalushskaya 19, Moscow, U.S.S.R.

Ambartsumian, Victor Amazaspovich, 1959 (2), Burakan Astronomical Observatory, Erevan, Armenia, U.S.S.R.

Bailey, Sir Edward, 1944 (6), 76 Hampstead Way, London, N. W. 11, England

Bartlett, Sir Frederic Charles, 1947 (12), 161 Huntingdon Road, Cambridge, England

Best, Charles Herbert, 1950 (9), Banting and Best Department of Medical Research, University of Toronto, Toronto, Canada

Bohr, Niels, 1925 (3), Institute for Theoretical Physics, Blegdamsvej 15, Copenhagen, Denmark

Bordet, Jules, 1935 (10), Pasteur Institute, Rue du Remorqueur, 28, Brussels, Belgium

Born, Max, 1955 (3), Marcard Strasse 4, Bad Pyrmont, Germany

Bragg, Sir William **Lawrence,** 1945 (3), The Royal Institution, 21 Albemarle Street, London, W. 1, England

de Broglie, Prince Louis, 1948 (3), 94 Rue Perronet, Neuilly-sur-Seine, France

Bullard, Sir Edward Crisp, 1959 (13), Madingley Rise, Madingley Road, Cambridge, England

Burnet, Sir Macfarlane, 1954 (10), The Walter and Eliza Hall Institute of Medical Research, Melbourne, Australia

Caso, Alfonso, 1943 (11), Av. Sudderman No. 312, Col. Chapultepec-Morales, Mexico 5, D. F.

Chapman, Sydney, 1946 (13), High Altitude Observatory, Boulder, Colorado, U. S. A.

Dale, Sir Henry Hallett, 1940 (9), The Wellcome Trust, 52 Queen Anne Street, London, W.1, England

Dirac, Paul Adrien Maurice, 1949 (1), Department of Mathematics, St. John's College, Cambridge, England

Eskola, Pentti Eelis, 1951 (6), Helsinki University, Snellmanink. 5, Helsinki, Finland

Fisher, Sir Ronald Aylmer, 1948 (8), Department of Genetics, University of Cambridge, 44 Storey's Way, Cambridge, England

von Frisch, Karl, 1951 (8), The Zoological Institute, University of Munich, Munich, Germany

Geijer, Per, 1958 (6), Agnevaegen 5, Djursholm I, Sweden

Gregory, Frederick Gugenheim, 1956 (7), Imperial College of Science and Technology, London, S.W. 7, England

Hadamard, Jacques, 1926 (1), 12 Rue Emile Faguet, Paris XIV, France

Hartmann, Max, 1959 (8), Max Planck Institute for Biology, Tübingen, Germany

Hill, Archibald Vivian, 1941 (9), 16 Bishopswood Road, Highgate, London, N.6, England

Hodge, William Vallance Douglas, 1959 (1), The Master's Lodge, Pembroke College, Cambridge, England

Hopf, Heinz, 1957 (1), Swiss Federal Institute of Technology, Alte Landstrasse 37, Zurich, Switzerland

Houssay, Bernardo Alberto, 1940 (9), Viamonte 2790, Buenos Aires, Argentina

Jeffreys, Sir Harold, 1945 (13), St. John's College, Cambridge, England

Jones, Sir Harold Spencer, 1943 (2), 40 Hesper Mews, London, S.W. 5, England

Kapitza, Peter Leonidovich, 1946 (3), Institute for Physical Problems, Academy of Sciences of the U.S.S.R., Moscow, U.S.S.R.

Karrer, Paul, 1945 (5), University of Zurich, Zurich, Switzerland

Kihara, Hitoshi, 1958 (7), National Institute of Genetics, Misima, Japan

Krishnan, Sir Kariamanikkam Srinivasa, 1956 (3), National Physical Laboratory of India, Hillside Road, New Delhi 12, India

von Laue, Max, 1958 (3), Fritz Haber Institute of The Max Planck Society, Faradayweg 4, Berlin-Dahlem, Germany

Levi, Giuseppe, 1940 (8), Instituto di Anatomia Umana, Corso Massimo D'Azeglio, 52, Turin, Italy

Lim, Robert K. S., 1942 (9), The Miles-Ames Research Laboratory, Elkhart, Indiana, U. S. A.

Lindblad, Bertil, 1955 (2), Stockholm Observatory, Saltsjöbaden, Sweden

Lwoff, André, 1955 (9), Institut Pasteur, Paris XV, France

Michotte, Albert Edouard (Baron Michotte van den Berck), 1956 (12), University of Louvain, Louvain, Belgium

Mott, Nevill Francis, 1957 (3), University of Cambridge, Cavendish Laboratory, Free School Lane, Cambridge, England

Oort, Jan Hendrik, 1953 (2), Observatory of Leiden, Leiden, The Netherlands

Penfield, Wilder, 1953 (10), Montreal Neurological Institute, 3801 University Street, Montreal 2, Canada

Pérès, Joseph Jean Camille, 1956 (1), University of Paris, Paris V, France

Pieron, Henri, 1949 (12), Institute of Psychology, University of Paris, Paris, France

Portevin, Albert M. G. R., 1954 (4), 21, Boulevard de Beauséjour, Paris XVI, France

Reichstein, Tadeus, 1952 (5), Organisch-chemische Anstalt, St. Johanns-Ring 19, Basel, Switzerland

Renner, Otto, 1954 (7), The Botanical Institute, University of Munich, Munich, Germany

Robinson, Sir Robert, 1934 (5), 170 Piccadilly, London, W. 1, England

Ruzicka, Leopold, 1944 (5), Department of Organic Chemistry, Institute of Technology, Zurich, Switzerland

Southwell, Sir Richard Vynne, 1943 (4), The Old House, Trumpington, Cambridge, England

Steacie, Edgar William Richard, 1957 (5), National Research Council, Ottawa, Canada

Svedberg, The, 1945 (5), Fysikalisk-Kemiska Institutionen, Uppsala University, Uppsala, Sweden

Taylor, Sir Geoffrey Ingram, 1945 (1), Trinity College, Cambridge, England

Theorell, Axel Hugo, 1957 (14), Nobel Institute of Medicine, Solnavagen 1, Stockholm 60, Sweden

Tiselius, Arne W. K., 1949 (14), Institute of Biochemistry, Uppsala University, Uppsala, Sweden

Todd, Sir Alexander Robertus, 1955 (5), University of Cambridge, University Chemical Laboratory, Pembroke Street, Cambridge, England

Vallée-Poussin, C. de la, 1929 (1), 42, Avenue du Houx, Boitsfort, Belgium

Vening Meinesz, Felix Andries, 1939 (13), Potgieterlaan 5, Amersfoort, The Netherlands

Watson, D. M. S., 1938 (8), University College, Gower Street, London, W.C.1, England

Winge, Öjvind, 1949 (14), Department of Physiology, Carlsberg Laboratory, Copenhagen (Valby), Denmark

Yukawa, Hideki, 1949 (3), Yukawa Hall, Kyoto University, Kyoto, Japan

Number of Foreign Associates July 1, 1959: 62.

SECTIONS

(1) *Mathematics*—34 members

Albert, A. A., *Chairman* (1961)
Ahlfors, L. V.
Alexander, J. W.
Artin, E.
Bell, E. T.
Bochner, S.
Brauer, R. D.
Courant, R.
Doob, J. L.
Douglas, Jesse
Eilenberg, S.
Eisenhart, L. P.
Evans, G. C.
Friedrichs, K. O.
Gödel, Kurt
Hille, Einar
Jacobson, Nathan
Lefschetz, Solomon
Mac Lane, Saunders
McShane, E. J.
Montgomery, Deane
Moore, R. L.
Morse, Marston
Murnaghan, F. D.
Shannon, C. E.
Smith, Paul A.
Steenrod, N. E.
Stone, M. H.
Thomas, T. Y.
Veblen, Oswald
Walsh, J. L.
Whitney, Hassler
Whyburn, G. T.
Zariski, O.

Foreign Associates

Alexandroff, P. A.
Dirac, P. A. M.
Hadamard, Jacques
Hodge, W. V. D.
Hopf, Heinz
Pérès, Joseph
Taylor, Sir Geoffrey
Vallée-Poussin, C. de la

(2) *Astronomy*—30 members

Mayall, N. U., *Chairman* (1962)
Abbot, C. G.
Babcock, H. D.
Babcock, H. W.
Bowen, I. S.
Brouwer, Dirk
Chandrasekhar, S.
Clemence, G. M.
Goldberg, Leo
Greenstein, J. L.
Joy, A. H.
Kuiper, G. P.
McMath, R. R.
Menzel, D. H.
Merrill, P. W.
Minkowski, R. L.
Mitchell, S. A.
Morgan, W. W.
Nicholson, S. B.
Ross, F. E.
Schwarzschild, M.
Seares, F. H.
Shapley, Harlow
Slipher, V. M.
Spitzer, Lyman, Jr.
Stebbins, Joel
Struve, Otto
Whipple, F. L.
Whitford, A. E.
Wilson, Ralph E.

Foreign Associates

Ambartsumian, V.
Jones, Sir H. Spencer
Lindblad, Bertil
Oort, Jan Hendrik

(3) *Physics*—86 members

Loomis, F. W., *Chairman* (1960)
Allison, S. K.
Alvarez, L. W.
Anderson, C. D.
Bacher, R. F.
Bainbridge, K. T.
Bardeen, John
Beams, J. W.
Bethe, H. A.
Birge, R. T.
Bleakney, Walker
Bloch, Felix
Bonner, T. W.
Bradbury, N. E.
Brattain, W. H.
Breit, Gregory
Bridgman, P. W.
Brillouin, Leon
Brode, R. B.
Coblentz, W. W.
Compton, A. H.
Condon, E. U.
Dennison, D. M.
Deutsch, M.
DuBridge, L. A.
DuMond, J. W. M.
Dunning, J. R.
Epstein, P. S.
Feynman, R. P.
Fowler, W. A.
Franck, James
Gamow, George
Goldhaber, M.
Goudsmit, S. A.
Herb, R. G.
Hofstadter, R.
Houston, W. V.
Hull, A. W.
Kemble, E. C.
Kent, R. H.
Kerst, D. W.
Kittel, C.
Kusch, P.
Lamb, W. E., Jr.
Land, E. H.
Lauritsen, C. C.
Marshak, R. E.
Mayer, M. G.
McMillan, E. M.
Meggers, W. F.
Morse, P. M.
Mulliken, R. S.
Nier, A. O. C.
O'Brien, Brian
Oppenheimer, J. R.
Panofsky, W. K. H.
Purcell, E. M.
Rabi, I. I.
Ramsey, N. F.
Robertson, H. P.
Rossi, Bruno
Schiff, L. I.
Schwinger, Julian
Segrè, E.
Seitz, Frederick
Serber, R.
Simpson, J. A.
Slater, J. C.
Stern, Otto
Street, J. C.
Teller, Edward
Thomas, L. H.
Townes, C. H.
Tuve, M. A.
Uhlenbeck, G. E.
Van Vleck, J. H.
Webster, D. L.
Weisskopf, V. F.
Wentzel, Gregor
Wheeler, J. A.
Wigner, E. P.
Wilson, Edwin B.
Wilson, Robert R.
Wu, C. S.
Zacharias, J. R.
Zachariasen, W. H.

Foreign Associates

Bohr, Niels
Born, Max
Bragg, Sir Lawrence
de Broglie, Prince Louis
Kapitza, P. L.
Krishnan, Sir K.
von Laue, M.
Mott, N. F.
Yukawa, Hideki

(4) *Engineering*—45 members

Fisk, J. B., *Chairman* (1962)
Bain, E. C.
Benedict, Manson
Bode, H. W.
Briggs, L. J.
Buckley, O. E.
Bush, Vannevar
Chipman, John
Cochrane, E. L.
Curme, G. O., Jr.
Den Hartog, J. P.
Draper, C. S.
Dryden, H. L.
Foote, P. D.
Fletcher, Harvey
Gibbs, W. F.
Gilliland, E. R.
Greenewalt, C. H.
Hoover, Herbert
Hunsaker, J. C.
Jeffries, Zay
Kelly, M. J.
Lewis, W. K.
Loomis, A. L.
Mehl, R. F.
Murphree, E. V.
Olson, Harry F.
Pierce, J. R.
Raymond, A. E.
Savage, J. L.
Sherwood, T. K.
Shockley, W.
Slepian, Joseph
Smith, C. S.
Soderberg, C. R.
Stratton, J. A.
Suits, C. G.
Terman, F. E.
Thomas, C. A.
Timoshenko, Stephen
von Kármán, T.
Wilson, Robert E.
Zener, Clarence
Zinn, W. H.
Zworykin, V. K.

Foreign Associates

Portevin, Albert M. G. R. Southwell, Sir Richard V.

(5) *Chemistry*—89 members

Pitzer, K. S., *Chairman* (1962)
Adams, Roger
Badger, R. M.
Bartlett, P. D.
Bolton, E. K.
Brewer, Leo
Brode, W. R.
Brown, H. C.
Calvin, Melvin
Conant, J. B.
Cope, A. C.
Craig, L. C.
Crawford, Bryce, Jr.
Daniels, Farrington
Debye, Peter
Doty, Paul
Elderfield, R. C.
Emmett, P. H.
Eyring, Henry
Ferry, John D.
Fieser, L. F.
Flory, P. J.
Folkers, Karl
Fuoss, R. M.
Fuson, R. C.
Gates, M.
Giauque, W. F.
Gilman, Henry
Hammett, L. P.
Harned, H. S.
Hauser, C. R.
Hendricks, S. B.
Hildebrand, J. H.
Hirschfelder, J. O.
Hornig, D. F.
Jacobs, W. A.
Johnson, J. R.
Johnson, W. S.
Keyes, F. G.
Kimball, G. E.
Kirkwood, J. G.
Kistiakowsky, G. B.
Kolthoff, I. M.
Kraus, C. A.
La Mer, V. K.
Leonard, N. J.
Libby, W. F.
Lind, S. C.
Longsworth, L. G.
Lucas, H. J.
MacInnes, D. A.
Marvel, C. S.
Mayer, J. E.
McElvain, S. M.
Mees, C. E. K.
Newman, M. S.
Niemann, C. G.
Noyes, W. A., Jr.
Onsager, Lars
Pauling, Linus
Roberts, John D.
Rodebush, W. H.
Rossini, F. D.
Scatchard, George
Schlesinger, H. I.
Seaborg, G. T.
Shedlovsky, Theodore
Sheehan, J. C.
Smith, L. I.
Smyth, C. P.
Spedding, F. H.
Stockmayer, W. H.
Tarbell, D. S.
Taube, Henry
Tishler, Max
Urey, H. C.
Warner, J. C.
Westheimer, F. H.
Williams, J. W.
Williams, Robert R.
Williams, Roger J.
Wilson, E. Bright, Jr.
Winstein, Saul
Wolfrom, M. L.
Woodward, R. B.
Wyckoff, R. W. G.
Yost, D. M.
Young, W. G.
Zimm, B. H.

Foreign Associates

Karrer, Paul
Reichstein, Tadeus
Robinson, Sir Robert
Ruzicka, Leopold
Steacie, E. W. R.
Svedberg, The
Todd, Sir Alexander

(6) *Geology*—35 members

Woodring, W. P., *Chairman* (1960)
Abelson, P. H.
Allen, E. T.
Anderson, C. A.
Birch, Francis
Blackwelder, Eliot
Bradley, W. H.
Bramlette, M. N.
Bucher, W. H.
Buddington, A. F.
Buerger, M. J.
Chaney, R. W.
Cloos, Ernst
Day, A. L.
Dunbar, C. O.
Gilluly, James
Gregory, W. K.
Hess, H. H.
Hewett, D. F.
Hubbert, M. King
Kelley, W. P.
Knopf, Adolph
Krauskopf, K. B.
Larsen, E. S., Jr.
Longwell, C. R.
Lovering, T. S.
Mead, W. J.
Nolan, T. B.
Rubey, W. W.
Russell, R. J.
Schairer, J. F.
Simpson, G. G.
Turner, F. J.
Williams, Howel
Yoder, H. S., Jr.

Foreign Associates

Bailey, Sir Edward
Eskola, Pentti
Geijer, Per

(7) *Botany*—43 members

Riker, A. J., *Chairman* (1962)
Anderson, Edgar
Barker, H. A.
Beadle, G. W.
Blinks, L. R.
Bonner, David M.
Bonner, James
Brink, R. A.
Burkholder, P. R.
Chandler, W. H.
Clausen, Jens
Cleland, R. E.
Couch, J. N.
Delbrück, Max
Dodge, B. O.
Esau, Katherine
Fred, E. B
Goddard, D. R.
Hershey, A. D.
Hollaender, A.
Horsfall, J. G.
Jones, D. F.
Kaufmann, B. P.
Kunkel, L. O.
Lederberg, J.
Mangelsdorf, P. C.
McClintock, Barbara
Osterhout, W. J. V.
Raper, K. B.
Rhoades, M. M.
Robbins, W. J.
Sax, Karl
Sinnott, E. W.
Skoog, Folke
Smith, Gilbert M.
Stakman, E. C.
Stebbins, G. L.
Thimann, K. V.
Van Niel, C. B.
Walker, J. C.
Went, F. W.
Wetmore, R. H.
Wilson, P. W.

Foreign Associates

Gregory, F. G.
Kihara, H.
Renner, Otto

(8) *Zoology and Anatomy*—53 members

Demerec, Milislav, *Chairman* (1961)
Bartelmez, G. W.
Bigelow, H. B.
Bloom, William
Bodenstein, D. H.
Bodian, David
Burns, R. K.
Castle, W. E.
Cleveland, L. R.
Colbert, E. H.
Corner, G. W.
Danforth, C. H.
Dobzhansky, Th.
Dunn, L. C.
Glass, Bentley
Hamburger, Viktor
Harrison, R. G.
Hartman, C. G.
Harvey, E. N.
Haskins, C. P.
Herrick, C. J.
Hisaw, F. L.
Holtfreter, J.
Hubbs, C. L.
Hutchinson, G. E.
Irwin, M. R.
Jacobs, M. H.
Kellogg, Remington
Lerner, I. M.
Lewis, W. H.
Mayr, Ernst
Metz, C. W.
Miller, A. H.
Mirsky, A. E.
Muller, H. J.
Nicholas, J. S.
Painter, T. S.
Patterson, J. T.
Petrunkevitch, A.
Riddle, Oscar
Romer, A. S.
Schmitt, F. O.
Schrader, Franz
Smith, Philip E.
Sonneborn, T. M.
Stern, Curt
Sturtevant, A. H.
Twitty, V. C.
Weiss, Paul
Wetmore, Alexander
Willier, B. H.
Wright, Sewall
Zirkle, R. E.

Foreign Associates

Fisher, Sir Ronald
von Frisch, Karl
Hartmann, Max
Levi, Giuseppe
Watson, D. M. S.

(9) *Physiology*—33 members

Bard, Philip, *Chairman* (1960)
Astwood, E. B.
Aub, Joseph C.
Brink, Frank, Jr.
Bronk, Detlev W.
Cole, K. S.
Cournand, André
Davis, Hallowell
Erlanger, Joseph
Evans, H. M.
Fenn, W. O.
Forbes, Alexander
Gasser, H. S.
Gerard, R. W.
Hartline, H. K.
Hastings, A. B.
Landis, E. M.
Lloyd, D. P. C.
Loeb, R. F.
Long, C. N. H.
Lorente de Nó, R.
Magoun, H. W.
Mann, Frank
Marshall, E. K., Jr.
Meek, W. J.
Pitts, R. F.
Redfield, A. C.
Richards, A. N.
Richards, D. W.
Schmidt, C. F.
Smith, Homer W.
Visscher, M. B.
Wiggers, C. J.

Foreign Associates

Lord Adrian
Best, C. H.
Dale, Sir Henry
Hill, A. V.
Houssay, B. A.
Lim, Robert K. S.
Lwoff, André

(10) *Pathology and Microbiology*—41 members

Paul, John R., *Chairman* (1960)
Armstrong, Charles
Bailey, P.
Blalock, Alfred
Cannon, P. R.
Castle, W. B.
Coggeshall, L. T.
Cole, Rufus
Dalldorf, Gilbert
Dingle, J. H.
Dochez, A. R.
Dragstedt, L. R.
Dubos, R. J.
Enders, J. F.
Francis, Thomas, Jr.
Goebel, W. F.
Heidelberger, Michael
Horsfall, F. L., Jr.
Huggins, C. B.
Little, C. C.
Loeb, Leo
Long, E. R.
MacLeod, C. M.
Maxcy, K. F.
McMaster, P. D.
Meyer, K. F.
Miller, C. P.
Opie, E. L.
Rich, A. R.
Rivers, T. M.
Robertson, O. H.
Rous, Peyton
Sabin, Albert B.
Shope, R. E.
Smadel, J. E.
Taliaferro, W. H.
Tillett, W. S.
Waksman, S. A.
Watson, C. J.
Whipple, G. H.
Wood, W. Barry, Jr.

Foreign Associates

Bordet, Jules
Burnet, Sir Macfarlane
Penfield, Wilder

(11) *Anthropology*—12 members

Kluckhohn, Clyde, *Chairman* (1960)
Albright, W. F.
Coon, C. S.
Haury, Emil W.
Herskovits, M. J.
Kidder, A. V.
Kroeber, A. L.
Lothrop, S. K.
Movius, H. L., Jr.
Shapiro, H. L.
Spier, Leslie
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